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Brain-Derived Neurotrophic Factor (BDNF) Modulation of Kv1.3 in the Olfactory Bulb

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BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF) MODULATION OF

Kv1.3 IN THE Olfactory Bulb

By

BEVERLY SHELLEY COLLEY

A Dissertation submitted to the
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I dedicate this to every person in the world who ever sought after an ideal but did not find it in their lifetime and to my family: Cyril, Luke, James and Dana. I also dedicate this to my mother Shirley, my grandmother Nelly and my greatgrandmother “Ma” all of whom I dearly love for the faith, love and hope they passed onto me.
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<th>Description</th>
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<tbody>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AU13</td>
<td>Kv1.3 antibody made in Auburn University by Dr. Debra Ann Fadool</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DIV</td>
<td>Days in vitro</td>
</tr>
<tr>
<td>ECL</td>
<td>Epi-chemiluminescence</td>
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<tr>
<td>EGTA</td>
<td>ethylene glycol bis (2-aminoethyl ether)-N, N, N'-tetraacetic acid</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>4G10</td>
<td>mouse monoclonal anti-phosphotyrosine</td>
</tr>
<tr>
<td>GTP</td>
<td>guanine triphosphate</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HEK 293</td>
<td>Human embryonic kidney 293</td>
</tr>
<tr>
<td>HRP</td>
<td>horse radish peroxidase</td>
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<tr>
<td>Hz</td>
<td>Hertz</td>
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<tr>
<td>K⁺</td>
<td>Potassium ion</td>
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<td>KCl</td>
<td>Potassium chloride</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>kDa</td>
<td>kiloDalton</td>
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<tr>
<td>Kir 3</td>
<td>Potassium inward rectifier channel 3</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-out</td>
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<tr>
<td>KTX</td>
<td>Kaliotoxin</td>
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<td>Kv1.3</td>
<td><em>Shaker</em> voltage gated ion channel (1) and sub-type 3</td>
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<td>Kv1.5</td>
<td><em>Shaker</em> voltage gated ion channel (1) and sub-type 5</td>
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<td>LTP</td>
<td>Long-term potentiation</td>
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<td>MgCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Magnesium chloride</td>
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<td>Margatoxin</td>
</tr>
<tr>
<td>mM</td>
<td>milliMolar</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential media</td>
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<tr>
<td>MW</td>
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<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
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<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
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<tr>
<td>NT-3</td>
<td>Neurotrophin-3</td>
</tr>
<tr>
<td>NT-4</td>
<td>Neurotrophin-4</td>
</tr>
<tr>
<td>OB</td>
<td>Olfactory bulb</td>
</tr>
<tr>
<td>OBN</td>
<td>Olfactory bulb neuron</td>
</tr>
<tr>
<td>P1</td>
<td>Postnatal day 1</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethysulphonylfluoride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>RTKs</td>
<td>Receptor tyrosine kinases</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Shc</td>
<td>Src homology 2 containing domain</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2 domain</td>
</tr>
<tr>
<td>SH3</td>
<td>Src homology 3 domain</td>
</tr>
<tr>
<td>SVZ</td>
<td>Subventricular zone</td>
</tr>
<tr>
<td>TEA+</td>
<td>Tetraethylammonium ion</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TrkB</td>
<td>Tropomyosin-related kinase B</td>
</tr>
<tr>
<td>$V_c$</td>
<td>command voltage</td>
</tr>
<tr>
<td>$V_{1/2}$</td>
<td>Voltage at half-activation</td>
</tr>
<tr>
<td>$V_h$</td>
<td>holding voltage</td>
</tr>
<tr>
<td>$V_m$</td>
<td>membrane voltage</td>
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ABSTRACT

Voltage-gated potassium ion channels such as Kv1.3 have a role in altering excitability of neurons. The neuron has to have a sophisticated mechanism to regulate the modulation, expression, turnover and distribution of ion channels. Ion channels, like Kv1.3, become crucial in affording the neuron one of a symphony of players that can strategically play their part in transmitting electrical and chemical signals into meaning. This dissertation uses electrophysiology and biochemistry to investigate how brain-derived neurotrophic factor (BDNF) and TrkB utilize a very simple aspect of the biochemistry of Kv1.3 to specifically modulate the biophysical properties, expression and turnover of Kv1.3. Acute BDNF application suppresses Kv1.3 current and results in phosphorylation of tyrosine residues 111-113, 137 and 449. There is a delicate balance of other downstream cellular components; N-Shc, Grb10 and PSD95 that disrupt this BDNF induced current suppression. N-Shc disrupts a post-phosphorylation event that usually leads to BDNF-evoked Kv1.3 current suppression, and N-Shc causes phosphorylated Kv1.3 to be retained in the membrane. Grb10 and PSD95 left-shift the voltage at half-activation of Kv1.3 and this effect may not be phosphorylation-dependent. Grb10 also reduces Kv1.3 expression and causes redistribution of membrane inserted Kv1.3. The presence of BDNF activated tropomyosin-related kinase B (TrkB) increases the phosphorylation of Kv1.3 tyrosine residues and increases Kv1.3 expression by two fold. TrkB also increases the half-life of Kv1.3 and this can account for the increase in protein expression. Kv1.5 is another member of the Shaker family but TrkB decreases the expression of Kv1.5. The insulin receptor (IR) is also a tyrosine kinase like TrkB however, IR decreases Kv1.3 expression and has no effect on Kv1.5 expression. These effects demonstrate that the TrkB and IR mediated regulation of Kv1.3 expression is not a promiscuous interaction of Shaker channels with receptor tyrosine kinases. Given the prominence of Kv1.3 in the olfactory bulb, one can hypothesize that the above interactions can play a part in modulating the function of Kv1.3 during development, learning and injury in the olfactory bulb. The neuron can utilize these interactions to regulate Kv1.3 and change how Kv1.3 contributes to shaping the neuron’s response.
Voltage-gated potassium ion channels

**Potassium ion channels.** Potassium ion (K\(^+\)) channels are pores in biological membranes that allow the passage of charged atoms or ions. The K\(^+\) channel family of proteins are tetrameric or made up of four subunits that come together in the membrane to form a passage or pore for potassium ions to pass through the membrane. Potassium channels are found in many cell types throughout the organism including excitable cells like nerve cells and traditional non-excitable cells like lymphocytes. K\(^+\) channels are said to be very ubiquitous as at least one is found in all fully sequenced genomes – eukaryotic, eubacterial and archael (Littleton and Ganetzky, 2000). The K\(^+\) channels are founders of the S4-superfamily, cyclic nucleotide-gated channels and Ca\(^{2+}\) and Na\(^+\) channels (Miller, 2000). The S4-superfamily is found primarily in eukaryotes and is mainly for electrical signaling.

Potassium channels have a highly conserved segment in their sequence that makes them easy to recognize; it is called the K\(^+\) channel sequence (Heginbotham et al., 1994). This sequence is what makes them potassium ion channels, as it codes for the portion of the protein structure that allows it to selectively filter for potassium ions to pass through the aqueous pore at rates close to diffusion (MacKinnon, 2003). In this dissertation you will learn about the modulation a voltage-gated potassium (Kv). Kv channel belong to a sub-family of K\(^+\) channels that respond to changes in voltage across the membrane and hence it they are voltage-gated ion channels. A voltage change occurs across the biological membrane when there is a physiological change in composition or balance of charge separation across the membrane. There are four Kv sub-families: *Shaker* or Kv1, *Shab* or Kv2, *Shaw* or Kv3 and *Shal* or Kv4. I have focused on the Kv1 sub-family.

**Shaker ion channels.** The *Shaker*-family of channels are denoted by a 1 and its members by 1.1 to 1.9, such as Kv1.3 and Kv1.5, where K stands for potassium and the v for voltage-
gated. The Shaker-family of ion channels were named Shaker because the DNA was first cloned from the chromosome of Drosophila melanogaster (fruit fly) containing a mutant potassium channel gene (Papazian et al., 1987). This mutation caused the flies to shake their legs under anesthesia and hence the name ‘shaker’. Shaker channels are tetrameric so they contain 4 α-subunits each consisting of 6 membrane spanning or transmembrane domains. The amino and carboxy termini are on the intracellular side of the membrane. These four subunits come together with four-fold symmetry to form a central ion conduction pathway (Doyle et al., 1998). The pore is constructed with the selectivity filter at the wide end facing the extracellular side and looks like an inverted teepee. The selectivity filter is narrow and contains chemical and structural properties that allow for the passage of K⁺ ions with high selectivity and rapid conduction. It contains the ‘signature sequence’, like all Kv channels, between the two most carboxy-terminal transmembrane helices (Heginbotham et al., 1994). The signature amino acid sequence reads TMxTVGYG, with minor variations.

**Structure and Biophysical properties of Shaker ion channels.** Shaker channels open their ‘gate’ and let potassium ions through their water filled pores (Doyle et al., 1998) when there is a change in the voltage difference across the membrane. In Shaker channels the fourth segment or S4 spanning the membrane contains 4 arginine residues at every third position that move across the membrane and constitute the gating current responsible for sensing voltage changes and gating the channel. These movements bring about large conformational changes of the channel within the membrane such that the inner helices obstructing the pore expand its intracellular diameter and open the channel.

The Shaker channels gradually stop conducting K⁺ ions by two main mechanisms: N-type or C-type inactivation. ‘N-type inactivation’ leads to spontaneous blocking of the pore of the channels upon maintained depolarization. It involves a conformational change by the plugging of the intracellular end of the open pore by the channel’s N terminus 20 or so residues (Miller, 2000). It is also termed the ‘ball-and-chain’ mechanism. Even though the Shaker tetramer can contain multiple inactivation domains a single inactivation domain is sufficient to confer N-type inactivation. It can be abolished by deletion of the N-terminus and is inhibited by intracellular pore blockers namely tetraethylammonium (TEA⁺) and its derivatives (Kurata and Fedida, 2005). The second mechanism is ‘C-type inactivation’, which produces transient K⁺ conductance.
of the channels despite a maintained stimulus. The pore is pinched shut at the narrow selectivity filter itself by a conformational change in the channel. It is sensitive to high levels of extracellular $\text{K}^+$ and can be prevented by the binding of extracellular tetraethylammonium ion (TEA$^+$) to the pore. C-type inactivation persists in *Shaker* channels when the inactivation ball is deleted and is normally slower than N-type inactivation (Yellen, 2002). Elevation of extracellular $\text{K}^+$ in both physiological and pathological ranges slows entry into the C-type state (Marom and Levitan, 1994). Therefore in states with elevated $\text{K}^+$, the fraction of inactivated channels is expected to decrease thereby increasing the inhibitory effectiveness of $\text{K}^+$ channel activation.

**Function of Kv1.3 and Implications for Electrical Excitability.** The biophysical properties of Kv1.3, like any other Kv1 channel, depend on the structure. These properties determine the electrical excitability of the cell. At rest a cell will only be processing events of previously long-lasting stimuli, day-to-day maintenance, survival and repair. When a neuron needs to interpret, code, respond to changes in its environment and translate these changes into existing brain circuitry and behavior, it will use ion channels like Kv1.3. The biophysical properties of Kv channels can be manipulated by cellular components for the cell to respond and alter electrical excitability of its membrane and hence function. The typical excitable cell has ion channels other than potassium channels but with a ratio of about 30:1 $\text{K}^+$ ions, inside the cell versus outside, Kv channels tend to stabilize the membrane potential at a value close to where Kv opening and closing rates are equal. Kv channels therefore tend to have minimal effect at the resting potential of the membrane, in the range of -60 to -80 mV depending on the cell type. For this reason, K channels can set the resting potential, keep fast action potentials short, terminate periods of intense activity, time inter-spike intervals during repetitive firing and tend to lower the effectiveness of excitatory inputs on a cell when they are open (Hille, 2001).

Another property of Kv1.3 that affects the excitability of the cell is the time it takes to close or inactivate after being subjected to depolarization. Kv1.3 inactivates or stops conducting $\text{K}^+$ ions slowly in response to prolonged depolarization. Depolarization of a cell is characterized by the membrane potential becoming more positive and it goes from resting all the way to persistent depolarization that may occur in response to tonic stimuli. Kv1.3 channels can potentially affect electrical excitability of cells because of this tendency to ‘stabilize’ the
membrane potential around their own equilibrium. However, the precise inactivation behavior of Kv1 channels can modify the extent and kind of effect the channel has on electrical excitability. The precise inactivation behavior of Kv1 channels is very much dependent on their subunit partners in forming the functional tetrameric channel \textit{in vivo}. The Kv1.1 subunit seems to be very ubiquitous in the brain and has been co-immunoprecipitated along with the Kv1.3 subunit suggesting they exist as heteromultimers (Coleman et al., 1999). The specific auxiliary β-subunit composition can determine the surface expression level of Kv1 potassium channels (Manganas and Trimmer, 2000) and affect the rate of inactivation (Kurata and Fedida, 2005).

The importance of the role of this channel throughout the body is vast and astounding. Kv1.3 regulates energy homeostasis, body weight and insulin sensitivity (Xu et al., 2003; Xu et al., 2004) and is considered a key player in the β-Amyloid peptide-induced cortical neuronal death observed in Alzheimer’s (Yu et al., 1998). Kv1.3 is also seen as a possible candidate for breakthrough drug treatment for Multiple Sclerosis (Chandy et al., 2004), diabetes (Desir, 2005) and as a new candidate to prevent neuronal death in Alzheimer’s disease (Yu et al., 1998). Remarkably, Kv1.3 has recently been discovered in lymphocyte mitochondria (Szabo et al., 2005) and TrkB has been discovered in mitochondria of human muscle and brain cells (Wiedemann et al., 2005).

\textit{Shaker} channels are implicated in the pathology of damage after stroke (Yu et al., 1997; Chi and Xu, 2000) and nerve injury (Kim et al., 2002). \textit{Shaker} channels are also considered targets for developing drugs to treat heart disease (Brendel and Peukert, 2003), to improve the outcome of hypoxia- and ischemia-induced neuronal death (Wei et al., 2003) and prevent CA1 hippocampal injury following transient forebrain ischemia (Huang et al., 2001). The modulation of this channel and its properties are of interest to me because of the very important role it has in olfaction (my main broader area of interest) and associative learning (Kourrich et al., 2001).

\textbf{Distribution of Kv1.3.} In the brain, the transcript for Kv1.3 is found differentially expressed during development and remains fairly persistent in the dentate gyrus and internal layer of the olfactory bulb (Kues and Wunder, 1992). Kv1.3 mRNA is also found highly expressed in the CA3 region in the stratum pyramidale and smaller amounts in the stratum radiatum of the hippocampus. The Kv1.3 subunit along with Kv1.1 was found through binding studies with Kaliotoxin (KTX- selective Kv1.1 and Kv1.3 channel blocker) in the olfactory bulb.
specifically the internal plexiform layer, granular layer, and mitral soma layer with moderate expression in the glomerular layer, the hippocampal CA3 subfield and the cerebellum. There were also high densities in the frontal cortex, septal region particularly the bed nuclei stria terminalis, the nuclei of the basal ganglia, within neuron clusters of the caudate putamen and most of the amygdaloid nuclei particularly in the amygdalo-hippocampal area. The highest levels of KTX binding sites were found in the neocortex, bed nuclei of the stria terminalis, most of the hypothalamus, dentate gyrus, central gray and parabrachial nuclei (Mourre et al., 1999) and microglia (Khanna et al., 2001). Mourre and colleagues data also suggested that in every structure containing intermediate to high densities of KTX binding sites, Kv1.1 or Kv1.3 mRNA (or protein) was detected, this will be significant in understanding the function of Kv1.3 channels in native cells discussed in our next paragraph as Kv1 or Shaker channels are found to mostly exist as heteromultimers. Grosse and colleagues (Grosse et al., 2000) found Kv1.3 α-subunit predominantly localized to the perikarya and axon of CA1-CA3 and granule cells of mouse hippocampal slices and culture. In slice they also observed some Kv1.3 antibody staining in the axons of granule cells (Grosse et al., 2000). Grosse and colleagues also show that except for the CA1-CA3 region of the hippocampus, Kv1.1 subcellular distribution overlaps with Kv1.3. The only difference is that Kv1.1 is also found in the axons of CA1-CA3 region whilst Kv1.3 is not. Kv1.1 was the first Kv channel to be observed at postnatal day 6 in CA1-CA3 and the dentate gyrus in slice and this was mimicked in culture in which Kv1.1 appeared after 10 DIV. Kv1.3 was first expressed in both pyramidal and granule cells between 12-14 DIV. All Kv1 channels were expressed by days 18, with Kv1.4 and Kv1.5 expressing the latest. Hence this is one example of how the electrical property of a cell’s membrane is controlled with development and can be used as a marker of the stage of development, the excitability of the membrane of the cell and circuits expressing these Kv1 channels.

**Modulation of Kv1.3 in the olfactory bulb.** Kv1.3 is highly expressed in the olfactory bulb and carries about 60-80% of the outward current in olfactory bulb neurons (Fadool and Levitan, 1998). Our laboratory has shown that Kv1.3 is predominantly expressed in the mitral and granule cell layers of the olfactory bulb in a developmentally regulated manner (Fadool et al., 2000). Hence mitral cells of the olfactory bulb are appropriate neurons in which to study the function of Kv1.3. Our laboratory was the first to demonstrate neurotrophic modulation of
Kv1.3 in second order neurons called mitral cells in the olfactory bulb. We observed a time- and sensory-dependent modulation of Kv1.3 by a neurotrophic factor. My investigation comprising Chapter 1, aimed to determine what was the mechanism of modulation and in the process I uncovered another level of modulation that I explored and which became the aim of Chapter 2.

**Neurotrophins**

**Classification of Neurotrophins.** The search for ‘neurotrophic factors’ started with classical embryological observations of Viktor Hamburger in 1952 (Lindsay, 1996). Brain-derived neurotrophic factor (BDNF) was the second neurotrophic factor isolated (Barde et al., 1982) after nerve growth factor. The neurotrophic hypothesis first put forward by Levi-Montalccini and Angletti in 1968 (Levi-Montalcini and Angletti, 1968) gained more credence with the discoveries of BDNF and neurotrophin-3 (NT-3). The hypothesis provided an explanation for how target fields influence the size of the neuronal populations that innervate them. The hypothesis states that “the survival of developing neurons depends on the supply of a neurotrophic factor that is synthesized in limiting amounts in their target field” (Davies, 1996).

**Brain–derived Neurotrophic Factor.** BDNF is a 27-kDa basic protein of non-covalently linked 13.5-kD subunits (Rosenthal et al., 1991). Barde and colleagues cloned and functionally expressed BDNF in 1989 (Leibrock et al., 1989) and found that BDNF mRNA was predominantly in the central nervous system. They also found that the protein sequence was structurally related to NGF (Leibrock et al., 1989). The rat BDNF gene has four 5'-exons (I-IV) each including its own promotor and combines with the 3' exon (V) to yield an mRNA coding for pre-pro-BDNF. A short stretch of exon V contains an alternative splice site, giving rise to two differentially spliced mRNA variants for each of the four transcripts. Each of these mRNAs is expressed in a tissue-specific and developmentally regulated manner (Timmusk et al., 1994; Timmusk et al., 1995). BDNF, apart from exerting its classical neurotrophic effects, exerts acute effects on synaptic transmission (Rose et al., 2004). Many of these effects are mediated by its modulation of ion channels properties such as a G-protein-activated inwardly rectifying potassium channel (Kir3) by tyrosine phosphorylation (Rogalski et al., 2000). Our laboratory
has shown that in cultured neurons from the olfactory bulb, acute BDNF application decreased Kv1.3 current amplitude in about 15 minutes (Tucker and Fadool, 2002). My first chapter explores this neuromodulation as I sought to identify which tyrosine residues on the channel needed to be present for BDNF to evoke current suppression of Kv1.3. I hypothesized that BDNF acting though its tyrosine kinase receptor would be capable of phosphorylating specific tyrosines of the channel and this may be part of the mechanism of current suppression. Hence, BDNF effects via TrkB, can target ion channels such as Kv1.3 to effect changes in electrical properties of the membrane and therefore affect other aspects of cell physiology important for growth, repair, survival and plasticity.

Extracellular BDNF levels must reach a certain threshold to induce long-term potentiation (LTP) in the hippocampus and in the neocortex. In addition, high frequency synaptic stimulation must be followed by sustained extracellular levels of endogeneous BDNF in order for long-lasting (>3 hours) LTP to occur. Also theta (≤100 hz - cyles per second, for a minute or less) burst stimulation is BDNF-dependent but tetanic (≥100 hz and for seconds to minutes) stimulation induced LTP is not solely dependent on BDNF (Lessmann et al., 2003).

BDNF’s effects is mediated by its receptor which is a transmembrane tyrosine kinase termed Trk (tropomyosin-related kinase). Each type of neurotrophin binds preferentially to one type of receptor over the others. NGF is the preferred ligand for TrkA; BDNF and Neurotrophin-4 (NT-4) both bind TrkB; and neurotrophin-3 (NT-3) has a high affinity for TrkC, although it is also able to signal through TrkA and TrkB (Lindsay, 1996). However, the first neurotrophin receptor isolated was the pan-neurotrophin receptor p75NTR, this receptor transmits both positive and negative signals. The Trk receptors transmit positive signals such as enhanced survival and growth (Kaplan and Miller, 2000), thereby serving as instruments of the classic roles of neurotrophins, differentiation, growth and survival.

**BDNF in the olfactory bulb.** In 1991, Guthrie and Gall reported finding BDNF mRNA primarily in the granule cell layer of rat olfactory bulb (Guthrie and Gall, 1991). In 1993, Deckner and colleagues found BDNF mRNA in low levels in mitral cells, the glomerular layer and the granule cell layer of rat olfactory bulb (Deckner et al., 1993). In cat, the signal was similar to rat except there were low levels in the mitral cells and more in the glomerular layer and granule cell layer of the rat. This may have been due to differences in design of the peptide
sequence for the probe and subtle differences in protocol and species. BDNF immunoreactivity was later found in mitral cells, periglomerular cells and the granule layer of rat olfactory bulb (Conner et al., 1997). Yan and colleagues also confirmed finding BDNF mRNA in rat olfactory bulb but did not give sublocalization details (Yan et al., 1997). Our laboratory found BDNF labeling throughout the external plexiform and granule cell layer of mouse olfactory bulb. When considering BDNF immunoreactivity, one must consider that labeling in a particular region or structure may not represent sites of synthesis but may represent pools of previously internalized BDNF that may have been released at some distance away or from a closer paracrine source. BDNF may also have autocrine actions and its signaling can occur at the membrane as well as chiefly after endocytosis of TrkB bound to BDNF. The endocytotic vesicles containing internalized TrkB bound to BDNF, leave the intracellular portion of the TrkB receptor and associated downstream signaling molecules to face the cytoplasm while the vesicles are being transported to various degradative pathways (Patapoutian and Reichardt, 2001). Chen and colleagues reported that in PC12 cells stably expressing FLAG-Trk receptors in the presence and absence of ligand, TrkB was predominantly sorted to the degradative pathway (Chen et al., 2005). They reported that TrkB receptors were rapidly internalized in the presence of ligand for 30 min at 37°C with an internalization half-life of 5 ± 1 minute (Chen et al., 2005). They also found that unlike TrkB, TrkA possessed an endocytotic sequence signal that was sufficient and necessary for rapid recycling of the TrkA receptors.

Infusion of BDNF (36 µg/day) into the olfactory bulb with a cannula and osmotic pump for 12 days resulted in a decrease in receptor protein in the olfactory bulb (Frank et al., 1997). This decrease was not accompanied by a decrease in mRNA levels in the olfactory cortex which is afferent to the olfactory bulb (Frank et al., 1997). This same group had previously investigated the effects of ligand mediated-regulation of TrkB using embryonic hippocampal and striatal neuronal culture. They found that addition of BDNF during an initial 24 hour period resulted in pronounced down-regulation of both TrkB protein and mRNA after 6 days. Intraventricular administration of BDNF also increases the number of newly generated neurons in the adult olfactory bulb (Zigova et al., 1998). In the olfactory bulb there is a steady stream of new neurons into the granule cell layer from the subventricular zone (SVZ) via the migratory stream (Luskin, 1993; Stewart et al., 1999; Stewart et al., 2002). BDNF can therefore promote the survival and renewal of the major inhibitory neuronal population in the olfactory bulb. We
have co-localized Kv1.3 and TrkB axonal/dendritic structures within the granule layer (B. K. Chandu, unpublished). One can hypothesize that BDNF-evoked modulation of Kv1.3 changes at various stages of development. BDNF modulation of Kv1.3 might also play a role in newly generated granule cell excitability, survival and incorporation of granule cell dendrites into existing circuitry in the olfactory bulb.

As rats age, the BDNF levels increase in the hippocampus (260% more) and decrease in the cerebral cortex. It is significantly lower in the septum, cerebral cortex, cerebellum and striatum by 30, 56, 52 and 52% respectively. There was no significant change in the wet weight (ng/g) for the olfactory bulb in rats or mice (Katoh-Semba et al., 1998). Intraventricular administration of BDNF (0.5 µl/hr, 12 µg BDNF/day) for 12 days into the right lateral ventricle of adult rat brain produced significantly more newly generated neurons in the olfactory bulb (Zigova et al., 1998). Given that BDNF mRNA is found in mitral cells, granule cells and periglomerular cells in the olfactory bulb, the pool of BDNF protein produced by these neurons can act as the trophic target for the migrating progenitors from the SVZ. Most of this type of work has been done in the hippocampus and prefrontal cortex where it is known that BDNF can enhance synaptic efficacy whereas hippocampal long-term potentiation (LTP) is impaired in BDNF knock-out (KO) mice (Lessmann et al., 2003; Lu, 2003). However, one study investigated the acute effects of exogeneously applied BDNF (200 ng/ml) to rat olfactory bulb one day old cultured slices and found that after six hours of BDNF the mean diameter of the dendritic processes (filapodia/spines) increased, but the length and density of the processes were not affected (Matsutani and Yamamoto, 2004). These changes were reported to be similar in both the external plexiform and granule layers. In slices treated for one hour with 0.5 ng and 5 ng of BDNF the changes were similar and detected as early as 30 minutes when 50 ng was applied (Matsutani and Yamamoto, 2004). In Chapter two, I report data showing that the granule layer contains Kv1.3 regions overlapping with TrkB. One can test the role of BDNF modulation of Kv1.3 in olfactory bulb circuit formation by recording the changes in dendritic morphology of mitral and granule cells in olfactory stimuli deprived, normal and over stimulated mice. These can be repeated in the presence and absence of Kv1.3 blockers. Kv1.3 knockout mice can be used as a possible control for the developmental contribution of Kv1.3 to shaping the circuitry and the morphological responsiveness of the dendrites.
Phosphorylation

**Protein Kinases and tyrosine phosphorylation.** Kinases are enzymes that catalyze or accelerate the rate of a biological reaction. Protein kinases belong to a special class of enzymes called phosphotransferases. They are also converter enzymes because they catalyze the ATP-dependent phosphorylation of serine, threonine or tyrosine hydroxyl groups in target proteins. Tyrosine kinases phosphorylate only tyrosine residues and occur only in multicellular organisms. They are very important for signaling pathways involved in cell-cell communication particularly as receptors for neurotrophins. There are basically two types of tyrosine kinases, cytosolic and membrane bound. Membrane bound tyrosine kinases are also called receptor tyrosine kinases (RTKs) and are most often receptors of hormones (Garret and Grisham, 1999). This dissertation will investigate a specific RTK and how its activation and subsequent phosphorylation of specific tyrosine residues on Kv1.3 alters the biophysical properties, expression and stability of the channel. It will also be demonstrated for the first time that a Shaker channel can reciprocally regulate the expression of two tyrosine kinases.

**Tropomyosin-related kinase B.** Tropomyosin-related kinase B (TrkB) receptors have a common extracellular structural organization that separates them from other membrane tyrosine kinases. They contain an immunoglobulin-like domain that (Huang and Reichardt, 2003) binds the preferred ligand, followed by a single transmembrane domain and a cytoplasmic domain that contains a tyrosine kinase domain in addition to several tyrosine-containing motifs similar to what is in other receptor tyrosine kinases (see Figure 3.1). Upon activation of the Trk receptor there is dimerization of the receptor. This brings about activation of the kinase, while phosphorylation of additional tyrosines creates docking sites for protein interactions with src homology 2 (SH2) domain containing proteins (Huang and Reichardt, 2003). The first gene to be identified that is capable of producing cell transformation was src (Tatosyan and Mizenina, 2000). SH2 domains bear homology to regions of src and are modulating regions of a protein that recognize a short amino acid sequence containing phosphotyrosine. These interacting proteins can then activate intracellular signaling events. Research has mainly focused on two phosphorylated tyrosines, Y490 and Y785. The details of how at least eight of the remaining cytoplasmic tyrosine residues contribute to Trk-mediated signaling remains largely unknown.
(Huang and Reichardt, 2003). There are currently three identified mammalian TrkB isoforms that bind neurotrophins: the full-length 145 kDa isoform, and two truncated isoforms of roughly 95 kDa, called T1 and T2, which both lack cytoplasmic tyrosine-kinase domains (Barbacid, 1994). Interestingly, the human gene encoding TrkB can generate only three major protein isoforms: the full length receptor, an isoform lacking the tyrosine kinase domain and a novel isoform lacking the tyrosine kinase domain but containing the Shc binding site. These three isoforms were only expressed in the brain (Stoilov et al., 2002).

**TrkB in the olfactory bulb.** In the olfactory bulb of rat and cat, trkB mRNA encoding full length TrkB was seen in the mitral and tufted cells of the olfactory bulb. Full-length TrkB receptor immunoreactivity was seen in the fila olfactoria (olfactory nerve), the epithelium and in olfactory sensory neurons (Deckner et al., 1993). Feinstein’s group found heavy labeling in the olfactory nerve layer and light labeling in the olfactory glomeruli and internal granular layer. They found intense labeling in the external tufted neurons in the periglomerular region and mitral cells, and the slices were all taken from rat (Yan et al., 1997). Deckner’s work also showed dense labeling in the glomerular layer, labeled cells outlining the glomeruli, moderate in the olfactory nerve layer, tufted and mitral cells and labeling the superficial granule cell layer but weaker in the deeper laminae of rat. Therefore, in cat Deckner found dense staining in the olfactory nerve layer and moderate in the glomerular but the rest of the labeling was similar to rat. So there is substantial overlap of TrkB labeling in the various layers of the olfactory bulb in different species but with some differences. I have carried out immunohistochemistry experiments to localize Kv1.3 and TrkB in the olfactory bulb of mice to demonstrate relevance of TrkB and Kv1.3 interaction in the olfactory bulb.

**Protein-Protein Interactions**

**Protein-protein interactions and adaptor proteins.** Extracellular signals are relayed from the cell membrane to intracellular targets by coordinating activities of membrane and cellular proteins. Specialized cellular proteins called adaptor proteins contain modules in their structure that allow them to form specific protein-protein interactions. The modules are domains that recognize a specific sequence in the target protein and allow signaling complexes to be
formed (Pawson and Scott, 1997). src homology 2 domains (SH2) are protein modules that recognize short, phosphopeptide motifs composed of phosphotyrosine. src homology 3 domains (SH3) bind to polyproline motifs. Several adaptor proteins are found in the olfactory bulb and I will focus on two, namely neuronal Shc and growth receptor binding protein 10 (Grb10) (Cook and Fadool, 2002).

**N-Shc adaptor protein.** Adaptor proteins, such as belonging to the Shc family, take part in downstream signaling and bind to phosphorylated tyrosines of activated receptors of growth factor- and cytokine receptor signaling. Neuronal Shc (N-Shc/ShcC/Rai) is a new member of the Shc family, that is expressed exclusively in the brain, compared to Shc that is present in all other non-neuronal tissue (O'Bryan et al., 1996). N-Shc contains two potential phosphotyrosine-binding domains, PTB and SH2 (see Figure 3.1). N-Shc can bind the activated TrkB receptor on stimulation with BDNF (Nakamura et al., 1996). Mice lacking the TrkB shc site, lose innervation of their sensory neurons (Postigo et al., 2002). Rai (N-Shc/ShcC) has recently been shown to regulate the neuronal adaptive response to environmental stresses (Troglio et al., 2004). The authors showed that, for Rai-/- mice, ischemia/reperfusion injury induced severe neurological deficits, increased apoptosis, the size of the infarct area and mortality. They demonstrated that Rai functions as a stress-response gene that increases phosphatidylinositol 3-kinase activation and Akt phosphorylation after hypoxic or oxidation insults. Recently, Miyamoto and colleagues reported that ShcC/N-Shc mutant mice exhibited superior ability in hippocampus-dependent spatial and nonspatial learning and memory (Miyamoto et al., 2005). Electrophysiological analyses revealed that hippocampal long-term potentiation in ShcC mutant mice was significantly enhanced, with no alteration of presynaptic function. The tyrosine phosphorylation of NMDA receptor subunits NR2A and NR2B was also increased, suggesting that ShcC mutant mice have enhanced NMDA receptor function in the hippocampus.

**Growth receptor binding protein.** Growth receptor binding protein (Grb) was initially cloned from an expression cDNA library through its binding to the tyrosine phosphorylated intracellular domain of the epidermal growth factor receptor (Ooi et al., 1995), hence its name. The binding was relatively weak so it was considered that the EGF receptor might not be the chief binding partner of Grb10. Grb10 has since been found to be a cellular partner of other
receptor tyrosine kinases such as the insulin receptor and other intracellular signaling mediators (Holt and Siddle, 2005). Grb10 (see Figure 3.1), like other members of its family, has a structure containing a proline rich N-terminal domain, a homology domain with mouse immunoglobulin gene 10 (MIG-10) including a Ras-associating (RA)-like domain, a pleckstrin homology region (PH), a C-terminal Src homology 2 (SH2) domain, and a receptor binding domain located between the PH and the SH2 domains (BPS). The PH domain is thought to facilitate localization to membranes through its interaction with phospholipids (Lemmon, 2003). Grb10 was found to differentially interact with the activated insulin receptor, insulin-like growth factor I receptor and the epidermal growth factor receptor via the BPS domain (He et al., 1998). Grb10 also expresses as various isoforms and they have been most studied for their function as cellular partners of the insulin receptor (IR) and insulin-like growth factor-I (IGF-I) receptor, particularly for their role in the action of insulin and growth and metabolism (Frantz et al., 1997; Dufresne and Smith, 2005; Mori et al., 2005). The specific contribution of Grb10 however, is highly dependent on the cellular context and that includes the balance of other signaling mediators that decide if altered levels of Grb10 and its various isoforms will enhance or restrain a particular response (Riedel, 2004; Holt and Siddle, 2005). In the murine family, Grb10 mRNA has been found highly expressed in insulin target tissues namely skeletal muscle, adipose tissue, heart and kidney. It was also detected in lower levels in the brain, lung and liver. In humans, the expression compares except it is also highly expressed in the pancreas with both the pancreas and skeletal muscle having several RNA transcripts. Grb10 mRNA was found in intermediate levels in cardiac muscle and brain. For a thorough review of Grb10 expression and functions, refer to Holt and Siddle (Holt and Siddle, 2005).

**N-Shc and Grb10 in the olfactory bulb.** Previous to the work done in our laboratory very little was known about the localization of N-Shc and Grb10 in the olfactory bulb. Our laboratory has shown that N-Shc and Grb10 are present in the rat olfactory bulb (Cook and Fadool, 2002) and their expression levels increase in the mouse olfactory bulb of Kv1.3 knockout mice (Fadool et al., 2004). This study shows the localization of Shc and Grb10 in the olfactory bulb and demonstrates another role Grb10 has in the brain. Unlike N-Shc, which is prevalent mainly in the brain and therefore has been studied in that context, the role of Grb10 has been studied primarily with respect to its role in the actions of insulin, growth and metabolism.
Brain-derived neurotrophic factor modulation of Kv1.3 and the olfactory bulb.

Neuromodulators are chemical substances usually composed of short peptide sequences that can be released in the brain from synaptic storage vesicles or otherwise act on neighbouring neurons to modulate or modify their responses to stimuli. It is not in a classical sense a neurotransmitter but in instances can be considered to act in a fast manner involving microseconds rather than seconds or minutes. In the strict sense of neuromodulation one usually thinks of a response that takes second to minutes and even days if its actions involve altering gene expression and remodeling of circuitry. In addition the neuromodulation may involve acute and chronic phases marked by the early effects of direct ligand binding to the receptor versus the later downstream effects of signaling from the receptor activation. Brain-derived neurotrophic factor was first identified not as a neuromodulator but as a neurotrophic factor supporting differentiation, growth and survival. Our laboratory explored various growth factors found in the olfactory bulb; NGF, BDNF and NT3, as possible neuromodulators of Kv1.3 in mitral cells. At first the experiments were done using rat olfactory bulb neurons. A Kv1.3 knockout mouse line became available and to carry out experiments using these mice required characterization of Kv1.3 currents in mice. Next, we needed to characterize BDNF-evoked modulation of Kv1.3 in mice. These two lines of experiments constitute the initial part of Chapter one and were carried out by Kristal Tucker. The rest of this dissertation stems from further investigation of the findings of Tucker and Fadool which demonstrated that Kv1.3 current in mitral cells cultured from the olfactory bulb was suppressed by acute application of BDNF and not NGF or NT3 (Tucker and Fadool, 2002). The suppression occurred within the first 15 minutes of BDNF application and lasted at least for the next 15 minutes. This BDNF-evoked Kv1.3 current suppression can be considered as a slow response as it takes place in a time frame of minutes. In the second part of Chapter one I tested the hypothesis that current suppression was tyrosine phosphorylation-dependent using Kv1.3 constructs containing specific tyrosine to phenylalanine mutations. I was able to show that the current suppression depended on the phosphorylation of
specific combinations of tyrosines. I also showed that there is an increase in Kv1.3 expression when co-transfected with TrkB. I continued to use the techniques of electrophysiology, Western blotting and immunochemistry to investigate the modulation of Kv1.3 by BDNF and TrkB from the cellular level to the level of the brain. The histochemistry and cytochemistry experiments were done in collaboration with Dr. Biju Chandu, a post-doctoral fellow in the laboratory. I use the olfactory bulb as my model of the brain because it contains neurons highly expressing Kv1.3 as well as TrkB. Our laboratory has also found that Kv1.3 knock-out (KO) mice have a heightened sense of smell and an increase in TrkB expression in their olfactory bulbs. We suspect that this relationship between Kv1.3 and TrkB may be important to understanding the observed changes in anatomy and function of the olfactory bulb in the KO mice and possibly even for normal structural development and function of the olfactory bulb in wildtype mice. My pursuit of this relationship became more meaningful to me and led to the hypotheses and experiments of Chapter two.

In Chapter two I hypothesized that perhaps the increase in Kv1.3 expression may be dependent on the kinase activity of TrkB and also on the availability of the tyrosine residues of Kv1.3 capable of being phosphorylated. I then tested the membrane current expecting to see an increase in current that would parallel the increase in overall expression of Kv1.3 in the cell. We had noted an increase in TrkB protein in the olfactory bulb of Kv1.3 knockout mice and I wondered if the relationship might be reciprocal. I also questioned whether this effect might be observed with another type of kinase, the insulin receptor kinase also found within the olfactory bulb and known to target its kinase activity to Kv1.3. I was interested in whether the increase in expression of channel protein might be a shared occurrence within the Shaker family of ion channels and hence tested Kv1.5. I chose Kv1.5 because of data I collected (not shown) to suggest that BDNF modulates Kv1.5 current in an opposite direction to Kv1.3; it is increased rather than suppressed. Perhaps the effect of TrkB on Kv1.5 expression might also be opposite to Kv1.3. I was particularly intrigued by the results because in microglia there is evidence showing a ‘switch’ from Kv1.5 current to Kv1.3 when microglia become activated. In trying to ascertain whether there was an increase in Kv1.3 in the membrane we used a Kv1.3 construct containing a surface myc epitope tag and another containing an N-terminus GFP tag. We observed that there was no increase in Kv1.3 expression with the construct containing the GFP tag. However, we did observe that there was increased c-myc labeling of mycKv1.3 in the
membrane suggesting the presence of a parallel increase similar to the increase in Kv1.3 channel expression in the cell. I then tested whether TrkB might be increasing the life or stability of Kv1.3 in the cell by using a protein synthesis inhibitor. The idea here was that, should TrkB be increasing the life or stability of Kv1.3 we should observe a sustained expression level or reduced rate of decrease of Kv1.3 protein in the presence of TrkB and cycloheximide, compared to in the absence of both TrkB and cycloheximide. I wanted to convince myself that the modulation of Kv1.3 by TrkB was relevant for olfactory bulb function as well as get an idea of where in the circuitry they may be co-localized so I did double-labeling experiments for Kv1.3 and TrkB in mouse olfactory bulb slices. I complimented the results of the histochemistry with co-immunoprecipitation from mouse olfactory bulb and showed that Kv1.3, Kv1.5, TrkB and IR do exist in protein-protein interactive states in the olfactory bulb.

In Chapter three I explored the possible function that adaptor proteins N-Shc and Grb10 can have in the olfactory bulb in respect to Kv1.3 and its modulation by BDNF. I tested their effects by co-expressing them in HEK293 along with Kv1.3 and TrkB. I also tested the importance of the TrkB shc site for the relief of current suppression by Grb10. I carried out co-localization experiments using histochemistry and found that the immunoreactivity of Shc and Grb10 overlap with Kv1.3 making it quite plausible that they can participate in BDNF-evoked modulation of Kv1.3. Next, I was able to co-immunoprecipitate Kv1.3 and Grb10 from transfected HEK293 cells and found that Grb10 can associate with Kv1.3 in the absence of TrkB. The ability of over-expressed Grb10 and its protein-protein interaction with Kv1.3 to relieve BDNF-evoked current suppression goes in line with previous findings that the exact role of Grb10 can depend on its expression level within cells. I was also able to co-immunoprecipitate Kv1.3 and N-Shc from the Kv1.3 membrane inserted pool in transiently transfected HEK 293 cells. These findings contribute toward understanding the possible ways that Kv1.3 can be modulated as part of a scaffold of interacting proteins in the membrane.
CHAPTER 1

Comparison of modulation of Kv1.3 channel by two receptor tyrosine kinases in olfactory bulb neurons of rodents ¹

Introduction

The activity of ion channels is dynamic, responding to intercellular signaling molecules and cytoplasmic factors via allosteric interactions and covalent modifications (Kaczmarek and Levitan, 1987; Levitan, 1994; Pawson and Scott, 1997). To understand the capacity of electrical signaling, one must ultimately elucidate the mechanisms by which channel proteins respond to biochemical changes at specific modulatory sites (Levitan, 1994). One such modulation occurs via phosphorylation. Phosphorylation of tyrosine residues, as a result of intercellular communication, modulates enzymatic activity and creates binding sites for the recruitment of downstream signaling proteins (Manning et al., 2002 2000). To date, several different types of tyrosine kinases have been demonstrated to produce short-term modulatory changes in neuronal excitability or ion channel function (Wang and Salter, 1994; Jonas and Kaczmarek, 1996; Bowlby et al., 1997; Sherwood et al., 1997; Fadool and Levitan, 1998; Berninger and Poo, 1999; Rogalski et al., 2000; Arnold et al., 2001; Huang and Reichardt, 2003).

The olfactory bulb (OB) represents a unique division of the central nervous system where a continual regeneration of basal cells produces neurogenesis in the adult. Growth factors, neurotrophins and their receptors are developmentally expressed postnatally but then persist in the adult OB at relatively high levels (Baskin et al., 1983; Hill et al., 1986; Gupta et al., 1992; Fadool et al., 2000; Tucker and Fadool, 2002). Thus it could be postulated that the growth factors

¹ This chapter was reprinted with permission (see Appendix A) from the original article Comparison of modulation of Kv1.3 channel by two receptor tyrosine kinases in olfactory bulb neurons of rodents. Receptors and Channels 10 (1) 25-36.
have a dual role in this system. They act in their traditional role as trophic factors during the growth and development of the olfactory system, but as members of receptor tyrosine kinase signaling cascades, they persist in the adult to modulate electrical activity via phosphorylation of ion channel proteins. Olfactory receptor neurons contained in the nasal epithelium must be regenerated approximately every thirty days (Graziadei and Monti-Graziadei, 1978) and modulation of electrical activity in the target OB could mediate circuit development, axonal pathfinding, or proper survivability and regeneration of these neurons.

The delayed rectifier, Kv1.3, a voltage-gated potassium (Kv) channel of the Shaker family, carries 60-80% of the K$^+$ current in rat OB neurons and has been found to be a molecular target for multiple phosphorylation by several tyrosine kinases including growth factors (Holmes et al., 1996a; Bowlby et al., 1997; Fadool et al., 1997; Fadool and Levitan, 1998 2000). Kv channels in the OB partly determine the membrane potential of a cell, regulate the level of neuronal excitability by influencing the duration of the action potential, determine the frequency of repetitive firing, and time the interspike interval (Jan and Jan, 1994). Long-term treatment of individuals with metabolic disorders or nerve injury demands an understanding of the modulatory events that may occur at the level of the ion channel to alter electrical signaling in individuals taking activators of tyrosine kinase receptors (diabetics, spinal injury patients).

Herein we characterize the suppression of the predominant Kv current in native OB neurons from the rat and mouse, by two different receptor tyrosine kinases (RTKs), insulin (IR) and neurotrophin B (TrkB) receptor kinase. Our goal of the study was to compare the mode of modulation of this K channel by two RTKs with different modes of activation and downstream signaling pathways. Culture conditions were established for mouse OB neurons based upon those previously known for rat as a basis for using the former in future studies employing gene-targeted deletions. Biophysical properties of Kv1.3 currents in mitral cells were characterized and compared across the two rodent models, including peak current amplitude, kinetics of inactivation and deactivation, voltage at half activation, and conductance. Acute application of the growth factors, insulin or BDNF to mitral cells of either rodent model, similarly induced current suppression without altering any other biophysical property measured. Also, in both rodent species, the modulation by the RTKs was eliminated by pretreatment with the Kv1.3-specific ion channel blocker, margatoxin, MgTx. Using a heterologous expression system, insulin and BDNF stimulation of cloned Kv1.3, co-expressed with one of the respective RTKs,
causes an increase in tyrosine phosphorylation of Kv1.3. It has been previously found that acute application of insulin to Kv1.3 plus insulin receptor (IR) kinase co-transfected cells has increased phosphorylation of Kv1.3 at Tyr\textsuperscript{111-113},\textsuperscript{137}, and\textsuperscript{479} (Fadool and Levitan, 1998; Fadool et al., 2000). Phosphorylation of these sites resulted in suppression of Kv1.3 current magnitude without altering channel kinetics (Fadool and Levitan, 1998). Now we report that co-expression of the channel with the neurotrophin receptor TrkB in the presence of stimulation with its principle ligand, brain-derived neurotrophic factor (BDNF), causes phosphorylation of a different combination of three tyrosine residues (Tyr\textsuperscript{111-113},\textsuperscript{137}, and\textsuperscript{449}) but results in the same electrophysiological effect. Interestingly, however, TrkB kinase co-expression with Kv1.3 induces an upregulation of total Kv1.3 channel protein that does not similarly occur in the presence of IR kinase. Thus while two RTKs cause suppression of Kv1.3 current by molecular targeting of multiple tyrosine residues in the C- and N-terminal domains, one of these (TrkB) may have a secondary response to alter channel expression even in the absence of ligand activation; likely representing a phosphorylation independent modulation.

Materials and Methods

**Solutions and Reagents.** Cultured olfactory bulb neuron (OBN) patch pipette solution contained (in mM): 145 KCl, 10 HEPES, 10 EGTA, 2 MgCl\textsubscript{2}, 0.20 NaATP, 0.5 GTP (pH 7.3). Cultured OBN bath recording solution contained (in mM): 150 NaCl, 5 KCl, 2.6 CaCl\textsubscript{2}, 2 MgCl\textsubscript{2}, 10 HEPES, and 100 nM tetrodotoxin (TTX) (pH 7.3). Patch pipette and bath recording solutions were formulated to maximize Kv1.3 whole-cell currents. Human embryonic kidney cell (HEK 293) patch pipette solution contained (in mM): 30 KCl, 120 NaCl, 10 HEPES, and 2 CaCl\textsubscript{2} (pH 7.4). HEK 293 cell recording bath solution contained (in mM): 150 KCl, 10 HEPES, 1 EGTA, and 0.5 MgCl\textsubscript{2} (pH 7.4). Cell lysis buffer with protease and phosphatase inhibitors (PPI solution) contained (in mM): 25 Tris (hydroxymethyl) aminomethane (pH 7.5), 250 NaCl, 5 EDTA, 1% Triton X-100, 1 sodium orthovanadate, 1 phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin A. Wash buffer contained (in mM): 25 Tris (pH 7.5), 250 NaCl, 5 EDTA, and 0.1% Triton X-100. TTX, a sodium channel blocker, was purchased from Calbiochem (La Jolla, CA). Margatoxin (MgTx), a Kv1.3 specific inhibitor, was a generous gift from Dr. Reid Leonard, Merck Research Laboratories. Human recombinant
brain derived neurotrophic factor (BDNF) was purchased from Promega (Madison, WI). Human recombinant insulin was purchased from Roche (Indianapolis, IN). Tissue culture reagents were purchased from Gibco/BRL (Gaithersburg, MD). All salts were purchased from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Houston, TX).

**cDNA Constructs and Antibodies.** Kv1.3 channel was subcloned into the multiple cloning region of pcDNA3 (Invitrogen) using the unique restriction site **HindIII**. IR cDNA was a gift from Dr. R. Roth (Liu and Roth, 1995) in the pECE vector and was subcloned into the multiple cloning region of pcDNA3 (Invitrogen) using the unique restriction site **SalI** and **XbaI**. TrkB cDNA was a generous gift from Dr. P. Barker (Atwal et al., 2000) in a CMX vector. All channel and kinase coding regions were downstream from a cytomegalovirus (CMV) promoter.

AU13, a rabbit polyclonal antiserum, was generated against a 46 amino acid sequence 478 MVIEEGMNHS AFPQTPFKTGNGSTATCTTNNPNDCVNIK KIFTDV 523 representing the unique coding region of Kv1.3 between the amino terminus and transmembrane domain 1. The purified peptide was produced by Genmed Synthesis (San Francisco, CA) and the antisera was produced by Cocalico Biologicals (Reamstown, PA). This antibody was used for immunoprecipitation (1:1000) and Western blot detection (1:1500) of Kv1.3. Tyrosine phosphorylated proteins were visualized by the anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology; Lake Placid, NY) and used at 1:1000 for Western blots.

**Primary Cell Culture.** Olfactory bulb neuron (OBN) primary cultures were prepared using a procedure modified from Huetther and Baughman (1986) as described previously (Fadool et al., 2000) for rat with minor adjustments for mouse. Briefly, olfactory bulbs were harvested from four Sprague Dawley rats (Simmonson, Kilroy, CA) or six B6129SF2/J mice (Jackson Laboratories, Bar Harbor, ME) on postnatal day 1 (P1) and placed into serum-free Dulbecco’s modified Eagle medium (dMEM) at 5% CO2, 37°C. The olfactory bulbs (OB) were incubated for 25-30 minutes for mice and 50-60 minutes for rats with cysteine-activated papain (200 U, Worthington Biochemicals, Lakewood, NJ) at 37°C, 5% CO2. To stop the enzymatic dissociation, OB were moved into dMEM containing 2% penicillin/streptomycin sulfate, 5% fetal bovine serum (FBS), and 5 mg/ml trypsin inhibitor (Roche) at 37°C, 5% CO2 for 10 minutes. Cells were mechanically dissociated by trituration using a graded-size series of fire-
polished siliconized Pasteur pipettes. The resulting neuron and glia suspension was plated onto poly-D-lysine hydrobromide (MW 49,300-53,000; Sigma) coated glass coverslips and grown in dMEM supplemented with 2% penicillin/streptomycin sulfate and 5% FBS. For mice OBN cultures, it was necessary to increase the density of the glial population by plating the neuron and glial suspension on an astrocyte feeder layer, previously established on the poly-D-lysine coated coverslips (Trombley and Blakemore, 1999). Ten micromolar cytosine arabinoside (Anderson et al.) was added to the media for 36 hours between day in vitro (DIV) 3-5 to stop overgrowth of dividing cells and promote better neuronal survivability.

**HEK 293 Cell Culture and Transient Transfection.** HEK 293 cells were maintained in Minimum Essential Medium (MEM), 2% penicillin/streptomycin, and 10% FBS (Gibco BRL). Before transfection, cells were grown to 95% confluency, dissociated with trypsin-EDTA (Sigma) and mechanical tritura tion, diluted in MEM to a concentration of 600 cells/ml, and plated on Corning dishes (Catalog # 25000, Fisher Scientific). cDNA was introduced into HEK 293 cells with a lipofectamine reagent (Gibco BRL) 3-5 days after passage as previously described (Fadool et al., 1997). Briefly, cells were transfected for 4-5 hours using 4.0 µg of each cDNA construct per 60 mm dish for biochemistry or 4-4.5 hours using 1.0 µg of each cDNA construct per 35 mm dish for physiology. Plasmid DNA with no coding insert (Fadool et al.) served as the control to equalize total µg of cDNA added to each dish. Cells were then harvested for biochemistry or used for patch-clamp recording approximately 30-48 hours post transfection.

**Electrophysiology of OBN Cultures.** OBN cultures were voltage-clamped in the whole-cell recording configuration. Electrodes were fabricated from Jencons glass (Hill et al., M15/10, Jencons Limited, Bedfordshire England), fire-polished to approximately 1 µm, and coated near the tip with beeswax to reduce the electrode capacitance. Pipette resistances were between 9 and 14 MΩ. All voltage signals were generated and data were acquired using pClamp 8 software in conjunction with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). The amplifier output was filtered at 5 kHz, digitized at 2-5 kHz, and stored for later analysis. Cells were held at -80 mV ($V_{m}$) and stepped to +40 mV ($V_c$) for a pulse duration of 400 msec, at a stimulating interval of 30 sec for peak outward current amplitude, inactivation, and deactivation measurements. Current-voltage relationships were assessed by holding the
neuron at -90 mV and stepping the voltage from -80 to +40 mV in 10 mV increments for 400 msec using a 30 to 60 second interpulse interval.

Acute stimulation of OBNs was achieved by bath application of 50 ng/ml BDNF or 10 µg/ml insulin after control measurements were acquired for approximately 10 minutes. Peak outward current amplitude, inactivation, deactivation and current-voltage relationships were measured pre-stimulation (time 0), 5, 15 and 30 minutes post stimulation. Differences in these biophysical measurements were analyzed between control (time 0) and post growth factor stimulation (time 15 min) groups within single cells by paired t-test with statistical significance at the 95% confidence level.

In experiments using margatoxin (MgTx) to block Kv1.3 specific current, voltage-clamped OBN in the whole-cell configuration were held at -90 mV and stepped to 40 mV for 1 second every 60 seconds. Currents were recorded for 3-5 minutes and then 100 pM of MgTx was bath applied. The current was recorded until the peak currents stabilized to the fully blocked level. Then the same neuron was treated by acute bath application of either 50 ng/ml of BDNF or 10 µg/ml insulin and recordings continued under the same voltage paradigm. Peak current amplitude was measured pre-MgTx stimulation, during block of Kv1.3 current by MgTx, and post growth factor stimulation.

All electrophysiological data were analyzed using pClamp 8.0 software in combination with the analysis packages Origin (MicroCal Software, Northampton, MA) and Quattro Pro (Borland International, Scotts Valley, CA). Data traces were subtracted linearly for leakage conductance. The inactivation of the macroscopic current was fit to the sum of two exponentials \( y = y_0 + A_1^{(-x/\tau_1)} + A_2^{(-x/\tau_2)} \) by minimizing the sums of squares, where \( y_0 \) was the Y offset, \( \tau_1 \) and \( \tau_2 \) were the inactivation time constants, and \( A_1 \) and \( A_2 \) were the amplitudes. The two inactivation time constants (\( \tau \)) were combined by multiplying each by its weight (\( A \)) and summing \( (\tau_{\text{inact}} = [(A_1 \tau_1) + (A_2 \tau_2)]/(A_1 + A_2)) \). The deactivation of the macroscopic current was fit similarly, but to a single exponential \( y = y_0 + A_1^{(-x/\tau_1)} \).

**Electrophysiology of HEK 293 Cells.** Macroscopic currents in cell-attached membrane patches were recorded 30-48 hr after transfection. The Kv1.3 channel expression was so robust that it was not possible to record whole-cell currents without saturating the amplifier. The diameter of the patch pipette, and hence number of ion channels sampled, was held uniform by
checking the bubble number of the pipette immediately after electrode fabrication and polishing (Mittman et al., 1987). Stimulation and analysis with insulin and BDNF was the same as described for OBNs, with the exception that the hormone/trophic factor was applied in the patch electrode.

**Immunoprecipitation.** For immunoprecipitation of Kv1.3 protein from transfected HEK 293 cells, cells were lysed in ice-cold PPI buffer. Lysis was continued on a Roto-Torque slow speed rotary (model 7637; Cole Palmer, Vernon Hills, IL) for 30 minutes at 4°C. The lysates were clarified by centrifugation at 14,000 g for 10 minutes at 4°C and precleared for 1 hour with 3 mg/ml protein A-sepharose (Amersham-Pharmacia, Newark, NJ), which was followed by another centrifugation step to remove the protein A-sepharose. Kv1.3 proteins were immunoprecipitated from the clarified lysates by overnight incubation at 4°C with α-AU13 (1:1000), followed by a 2 hour incubation with protein A-sepharose and centrifugation as above. The immunoprecipitates were washed 4 times with ice-cold wash buffer. Lysates and washed immunoprecipitates were diluted in sodium dodecyl sulfate (SDS) gel loading buffer (Sambrook and Russell, 2001) containing 1 mM Na$_3$VO$_4$ and stored at -20°C for subsequent analysis.

Whole-cell lysates or immunoprecipitated proteins were separated on 10% acrylamide gels by SDS-PAGE and electro-transferred to nitrocellulose blots. Blots were blocked with 5% nonfat milk (Biorad) and incubated overnight at 4°C in the presence of the antibody against Kv1.3 (α-AU13) or antiphosphotyrosine antibody (α-4G10). The blots were then incubated with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit secondary antibody (1:4000) (Amersham-Pharmacia) or HRP-conjugated goat anti-mouse secondary antibody (1:4000) (Sigma) for 90 minutes at room temperature. Enhanced chemiluminescence (ECL; Amersham-Pharmacia) exposure on Fugi Rx film (Shashoua et al.) was used to visualize labeled proteins. The film autoradiographs were analyzed by densitometry using a Hewlett-Packard Photosmart Scanner (model 106-816, Hewlett Packard, San Diego, CA) in conjunction with Quantiscan software (Biosoft, Cambridge, England).
Results

Comparison of Potassium Channel Properties Across Rat and Mouse in the Olfactory Bulb

Whole-cell Current Properties. Mouse was selected as the model to study the modulation of native Kv1.3 current properties due to the future perceived utility for studying transgenic animals. Unlike previous culture conditions known to support OB neurons from rat (Fadool et al., 2000; Tucker and Fadool, 2002), neurons harvested from mice did not survive well past two or three days in vitro (DIV) in the absence of an astrocyte feeder layer (see Methods). The total glial concentration contained in the mouse OB was apparently too low to support a co-culture of neuronal and glial cell types as is typically performed for rat. After establishing a confluent astrocyte feeder layer as an OB neuronal substrate, neurons from mice had the same longevity as that reported for rat (over 3 months) and the electrical stability of the patch recording increased, with most cells demonstrating stability over one hour.

Since there have been few reports describing electrophysiological properties of cultured mouse OB neurons as opposed to many describing that in rat, we initiated our study with a comparison between these two species. Cultured rat and mouse OB neurons were voltage-clamped in the whole-cell configuration. Neurons were held (V_h) at −90 mV and a family of current-voltage responses were generated by stepping the voltage (V_c) from −80 to +40 mV in 10 mV increments. The interpulse stimulus interval was at least 30 seconds to prevent cumulative inactivation of Kv1.3 current (Marom et al., 1993; Kupper et al., 1995). A representative family of current-voltage responses is shown in Figure 1.1A for each species. The plotted current-voltage relationship for a population of neurons demonstrates that voltage-gated properties of outward potassium currents are not inherently different between the two species (Figure 1.1B). This is best visualized by conversion of the voltage-activated currents to conductance to measure the corresponding voltage at half-activation (V_1/2) for each species (Figure 1.1C). As demonstrated in this figure and reported in Table 1.1, both the V_1/2 and the slope of the voltage-dependence (κ) were not significantly different between rat and mouse OB neurons (Student’s t-test, α ≤ 0.05).

Peak Current Magnitude and Kinetics of Inactivation Over Days In Vitro (DIV). Previous confocal imaging of Kv1.3 channel protein expression in rat OB neurons has
demonstrated detectable channel protein as early as DIV 2, which progressively and linearly increases over the first ten days in vitro (Fadool et al., 2000). Thus peak current magnitude and kinetics of inactivation were compared across rat and mouse from DIV 2-5. Neurons were held ($V_h$) at -90 mV and depolarized in a single step ($V_c$) from -80 to +40 mV with an interpulse interval of 1 minute. The inactivation time constant ($\tau_{\text{inact}}$) did not vary over DIV, nor was there a significant difference in the kinetics of inactivation between species (Table 1.1). As predicted, the peak current magnitude of the outward whole-cell current increased over DIV, presumably in register with an increase in Kv1.3 channel protein expression during growth of the neuron in vitro. The peak current magnitude for rat was nearly twice that found in mouse by DIV5 (Figure 1.1D). Given the restricted expression of Kv1.3 on the soma and dendrite of neurons and the notable size differences between rat and mouse OB neurons, it is not unexpected that the total current magnitude, but not current density, is greater in rat than in mouse.

Figure 1.1 Whole-cell current properties of cultured olfactory bulb (OB) neurons in rat and mouse. (A) OB neurons (Day 3 in vitro; DIV 3) were patch-clamped in the whole-cell configuration. Shown is a representative family of current responses for each species, where the
neuron was held (V_h) at -90 mV and stepped from -80 to +40 mV (V_c) in 10 mV increments for a duration of 400 msec using 30 second interpulse intervals between voltage stimulations.  (B) Current-voltage relation for a population of neurons that were stimulated as in Part A but using 20 mV increases in the depolarizing voltage steps.  Current was normalized to the current evoked at the +40 mV step.  Error bars represent the standard error of the mean (S.E.M.) for 6-10 trials per species.  ● = Rat and ■ = Mouse.  DIV 4.  (C) Conductance measurements were calculated for the voltage protocols performed in part (B) and plotted against voltage.  Maximum conductance was normalized to that of the +40 mV step.  Same notations and sample size part (B).  Solid line = best fit to a Boltzmann equation.  V_{1/2} = voltage at half-activation, is calculated from the Boltzmann fit.  (D) Peak current magnitude calculated from a simple voltage step (V_c) from –90 to +40 mV was plotted for 8-12 OB neurons at 2-5 DIV.  Same notations as in part (B).  Solid line = best fit to linear regression.

Table 1.1 Comparison of Biophysical Measurements in Whole-cell Recordings of Cultured Rat and Mouse OB Neurons (DIV 4-5)

<table>
<thead>
<tr>
<th>Species</th>
<th>Peak Current (pA)</th>
<th>( \tau_{\text{inactivation}} ) (ms)</th>
<th>( V_{1/2} ) (mV)</th>
<th>( \kappa )</th>
<th>Insulin % decrease</th>
<th>BDNF % decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>*2986 ± 428</td>
<td>221 ± 30</td>
<td>-22 ± 3</td>
<td>14.2 ± 1.4</td>
<td>25%</td>
<td>22%</td>
</tr>
<tr>
<td>Mouse</td>
<td>1567 ± 250</td>
<td>233 ± 54</td>
<td>-26 ± 7</td>
<td>18.6 ± 2.1</td>
<td>29%</td>
<td>*38%</td>
</tr>
</tbody>
</table>

The peak current was calculated as the maximum current evoked by a 400 ms depolarizing step from -90 to +40 mV.  The inactivation kinetics (\( \tau_{\text{inactivation}} \)) of the whole-cell current was calculated by an exponential fit to the inactivating portion of the current.  \( V_{1/2} \), the voltage at which half the channels were activated, was calculated by fitting normalized peak tail currents at different holding potentials to a Boltzmann Function (as in Figure 3).  The slope of this function or value for steepness of voltage dependence is reported as \( \kappa \).  * = Significantly different, Student’s \( t \)-test, \( \alpha \leq 0.05 \).  Arc-sin transformation for percentile data was used.  Sample size is denoted by parenthesis; values are mean ± S.E.M.

**Modulation of Mouse Olfactory Bulb Neurons by Activation of Receptor Tyrosine Kinases.**  Cultured mouse OB neurons were voltage-clamped in the whole-cell configuration with a \( V_h \) of -90 mV and voltage stepped (\( V_c \)) from -80 to +40 mV in 10 mV increments for a duration of 400 msec using a interpulse interval of 30 seconds.  A family of current-voltage relations was generated under control conditions (pretreatment) and 15 minutes following bath
application of 50 ng/ml BDNF or 10 µg/ml insulin. Representative current recordings prior and following neurotrophin or hormone application are found in Figures 1.2 and 1.3, respectively. Activation of the endogenous TrkB receptor kinase in these neurons (Tucker and Fadool, 2002) by the preferred ligand BDNF caused a significant decrease in peak current magnitude (810 ± 149 pA control, 325 ± 119 pA treated, paired $t$-test, $\alpha \leq 0.05$, $n = 5$). Similar activation of the endogenous insulin receptor kinase in these neurons (Gupta et al., 1992; Folli et al., 1994; Fadool et al., 2000) by insulin also caused a statistically significant decrease in peak current magnitude (1672 ± 237 pA control, 1227 ± 204 pA treated, paired $t$-test, $\alpha \leq 0.05$, $n = 9$). Tyrosine kinase-induced current suppression did not appear to be attributed to a change in voltage dependence of the Kv1.3 channel as indicated by a lack of shift in the current-voltage relation following application of BDNF or insulin (Figures 1.2 and 1.3, parts B). The proportional magnitude of current suppression by insulin in rat and mouse OB neurons was qualitatively the same and that by BDNF was roughly twice as great in mouse over that found in rat (Table 1.1).

**Modulation of Tyrosine Kinase-induced Current Suppression is Inhibited by Margatoxin Pretreatment (MgTx).** Even though the patch/bath solutions in our experiments were designed to favor Kv1.3 current expression in the OB neurons and reduced contribution by other potassium channel species such as calcium- or sodium-activated potassium channels, OB neurons do express two other members of the *Shaker* subfamily, namely that of Kv1.4 and Kv1.5. Thus it was important to ascertain whether receptor tyrosine kinases were acting upon Kv1.3 or other channels potentially contributing to potassium channel conductances. Neurons were held ($V_h$) at -90 mV and depolarized in a single step ($V_c$) from -80 to +40 mV for a duration of 400 msec with an interpulse interval of 1 minute. After permitting the neuron to stabilize for 3-5 minutes, the scorpion toxin margatoxin (MgTx) was bath applied to a final concentration of 100 pM. At this concentration, MgTx is selective as a blocker of Kv1.3 channel over that of other *Shaker* family members, and acts with a slow on rate of 10 minutes by blocking the lumen of the channel (Garcia-Calvo et al., 1993; Knaus et al., 1995). Cells were thus pre-treated with MgTx and current was monitored for 10-20 minutes, or until further current suppression was not visible. Then insulin or BDNF was applied as with a slow on rate of 10 minutes by blocking the lumen of the channel Garcia-Calvo et al., 1993; Knaus et al., 1995).
Figure 1.2 Brain-derived neurotrophic factor (BDNF) causes suppression of outward voltage-activated currents in mouse OB neurons. Same voltage protocols as described for Figure 1. (A) Representative family of current responses for a neuron prior (control) and following 15 minute bath application of 50 ng/ml BDNF (BDNF). (B) Current-voltage relation for a population of neurons. Error bars represent the S.E.M. for 6 neurons, prior (○) and following (●) bath application of the neurotrophin. Normalization as in Figure 1.1.
Cells were thus pre-treated with MgTx and current was monitored for 10-20 minutes, or until further current suppression was not visible. Then insulin or BDNF was applied as previously to test whether either was effective in modulating the remaining non- K\textsubscript{v}1.3 potassium current. As shown in Figure 1.4, pre-treated MgTx neurons did not respond to either insulin or BDNF treatment.
Figure 1.4 Margatoxin (MgTx) inhibits neuromodulation of OB neurons by the ligands for receptor tyrosine kinases, IR and TrkB. Plot of the peak current magnitude of representative neurons that were held ($V_h$) at -90 mV and then treated with the voltage protocol in Figure 3 using a 60 second interpulse interval. One hundred picomolar of MgTx, a Kv1.3-specific ion channel blocker, was applied at the arrow and current was monitored over time (●) until the blocked current no longer decayed (approximately 10-15 minutes). Current was normalized to the initial current recording at time 0. Then either 50 ng/ml BDNF (▲) or 10 µg/ml insulin (■) was added at the arrow and current was monitored for 20-30 min or the duration of the patch recording. Here current was normalized to the final MgTx-treated current level prior to treatment with tyrosine kinase activator. Records are representative for 5 such experiments for BDNF and 6 such experiments for insulin. *DIV 5.*

**Phosphorylation of Kv1.3 Channel Protein by BDNF-activation of TrkB kinase.** We have previously demonstrated that acute application of BDNF to adult rat OB causes an increase in tyrosine specific phosphorylation of native Kv1.3 (Tucker and Fadool, 2002). A more robust demonstration of the target of activated trkB kinase can be performed by elimination of phosphorylation and current suppression following mutagenesis of the channel. Therefore, human embryonic kidney (HEK 293) cells were transiently transfected with the cDNA coding for TrkB kinase plus wildtype Kv1.3 or mutant Kv1.3 channel whereby specific tyrosine residues
were altered by single point mutagenesis to phenylalanine at four strong recognition motifs for tyrosine specific phosphorylation (Tucker and Fadool). Thirty-six hours post transfection, cells were treated for eight minutes with 100 ng/ml BDNF or control vehicle as described in the methodology. Kv1.3 channel proteins were immunoprecipitated with the Kv1.3 specific antiserum, αAU-13, separated by SDS-PAGE, electro-transferred to nitrocellulose and then probed with anti-phosphotyrosine (anti-pY) specific antibody, α4G10, to determine any increase in tyr specific phosphorylation of the wildtype or mutant channel when acutely stimulated with BDNF. Representative immunoblots are shown in Figure 1.5A. Quantitative immunodensity calculations of 6-7 immunoprecipitations indicate that BDNF treatment causes a 4.2 ± 1.5 fold increase in wildtype channel phosphorylation (Figure 1.5B). This phosphorylation is lost when Y is changed to F at positions 111-113, 137, or 449 in the carboxyl or amino terminus of the channel. Y479F Kv1.3, however, retains a reduced ability to become phosphorylated by BDNF-activation of TrkB, indicating that this residue is not as important a target for BDNF-induced current suppression of Kv1.3 (Figure 1.5B). This site may be removed, and still retain a degree of phosphorylation and current suppression (see also Figure 1.7 below). Secondarily we noted that there is a significant increase in Kv1.3 channel expression when co-transfected with TrkB kinase even in the absence of BDNF (Figure 1.5C). Quantitative immunodensity calculations for this phosphorylation independent upregulation of the channel are shown in Figure 1.5D.

Lastly, loss of BDNF-induced Kv1.3 current suppression was sought for YYY111-113FFF, Y137F, and Y449F Kv1.3; all constructs that demonstrated loss of tyrosine phosphorylation by SDS-PAGE analysis under TrkB plus channel co-transfected conditions (Figure 1.6). HEK 293 cells were transiently transfected as described above but at cDNA concentrations appropriate for physiology (see Methods). Thirty-six hours post transfection, cells containing expressed wildtype and mutant Kv1.3 protein were screened for acute modulation of current by BDNF. Patch pipettes were tip-filled with control patch recording solution and then back-filled with 50 ng BDNF in order to access the TrkB receptor extracellular binding domain. Macroscopic currents were recorded from the cells by voltage-clamping in the cell-attached configuration. Patches were held at -90 mV (V_{h}) and stepped to a single depolarizing voltage of +40 mV (V_{c}) for a duration of 1000 msec with an interpulse interval of 45 seconds. Figure 8B demonstrates the first recorded trace following stabilization of the patch (Control) and that recorded 20 minutes after achieving the cell-attached configuration (BDNF)
Figure 1.5 Tyrosine phosphorylation of the Kv1.3 channel protein following BDNF stimulation. (A) Human embryonic kidney (HEK) 293 cells were transiently transfected with the cDNA for wildtype Kv1.3 plus TrkB receptor (WT Kv1.3) or cDNA for a Y to F point mutation in the channel plus TrkB receptor as indicated. Cells were acutely stimulated with control vehicle (-) or BDNF (+) supplemented media. Kv1.3 channel protein was immunoprecipitated (IP) from whole cell lysates and separated by SDS-PAGE. Proteins were electrotransferred to nitrocellulose and blotted (Blot) with the antiphosphotyrosine antibody, α-4G10 and visualized by species-specific hrp-conjugated secondary antibody. Arrows = Kv1.3 channel protein (55-68 kDa); band of immunoglobulin (IgG). (B) Total phosphorylated channel protein in part (A) was subjected to quantitative densitometry. Calculated pixel immunodensity under the control condition was normalized arbitrarily to 1.0. Mean immunodensity values are expressed as a ratio of BDNF-stimulated divided by control conditions. Each ratio value was calculated only within the same film autoradiograph to eliminate variability in epichemiluminense (ECL) exposure. Ratio value of 1.0 (dashed line) indicates no increase in phosphorylation. Number of transfections as indicated; Mean ± S.E.M., * = significantly different Students t-test, α ≤ 0.05, arc-sin transformation for percentage data. (C) Same techniques as in (A). Whole cell lysates were probed (Blot) with antisera against the Kv1.3 channel protein under BDNF unstimulated conditions. D) Histogram plot of the mean immunodensity values for four experiments as in part (C). Mean ± S.E.M., * = significantly different Students t-test, α ≤ 0.05, arc-sin transformation for percentage data.
for a cell transfected with TrkB plus wildtype Kv1.3 (Wildtype Kv1.3). BDNF significantly reduced the peak magnitude of the wildtype Kv1.3 macroscopic currents without alterations in the kinetics of inactivation or deactivation (Paired t-test, $\alpha \leq 0.05$, $n = X$) (Table 1.2). Figure 6C-F demonstrates similar representative recordings under the same experimental paradigm for cells co-transfected with TrkB + one of the Kv1.3 channel mutants. BDNF was ineffective in causing significant current suppression for any of the mutant Kv1.3 channels co-expressed with TrkB (Paired $t$-test, $\alpha \leq 0.05$, $n = X$) (Table 1.2) indicating that disruption in any one of the Tyr residues altered the collective phosphorylation sites to disrupt Kv1.3 modulation (Figure 1.7).

Data presented in the combined Figures 1.5 and 1.6 provide complementary evidence that multiple residues (Tyr$^{111-113}$, Tyr$^{137}$, and Tyr$^{449}$) are the molecular target for BDNF modulation of Kv1.3 via phosphorylation by TrkB kinase. Although not statistically significant, but perhaps representing site-specific functions, we noted that deletion of Y137 caused a slight change in the second Tau inactivation under BDNF-stimulated conditions (Figure 1.6D). Likewise deletion of Y449 caused a slight increase in current magnitude under BDNF-stimulated conditions (Figures 1.6E and 1.7).

**Discussion**

The activity of receptor tyrosine kinases (RTKs) is regulated during development of the nervous system. During pruning of neural circuits, during periods of injury or apoptosis, and in disease states, it can become uncoupled from normal regulatory processes or expression levels. Previous work has shown that activation of insulin receptor (IR) kinase and neurotrophin receptor B (TrkB) caused suppression of a voltage-gated potassium channel, Kv1.3. A direct route for indicating the role of tyr phosphorylation in the Kv1.3 modulation by RTKs is through mutagenesis. In the case of activation of IR kinase, the modulation was attributed to direct tyrosine phosphorylation of key residues in the amino and carboxyl terminal of the protein (Fadool et al., 2000). We now show that activation of TrkB kinase by its preferred ligand BDNF similarly modulates Kv1.3 by causing current suppression but utilizes a different combination of multiple tyrosine residues. It is interesting that both these RTKs appear to downregulate the activity of Kv1.3 via multiple phosphorylation given the inherent differences in their activation - IR kinase by binding insulin versus the dimerization of TrkB. Thus, this mechanistic difference
in receptor activation does not appear to play a role in the modulation of the potassium channel biophysics.

One possible interpretation for the role of multiple but differential phosphorylation of the channel by BDNF versus insulin, is that downstream signaling molecules of these RTKs may preferentially dock or become activated only when a specific combination of channel residues is phosphorylated. Both of the RTKs become phosphorylated at multiple Y residues in the cytoplasmic portions of the molecules, respectively, which then serve as specific recognition sites for discreet protein-protein interactions with, for example, SH2 containing adaptor proteins (review: Huang and Reichardt, 2003; i.e. Ishihara et al., 2003). It would not be surprising if the multiple Y sites along the channel acted similarly to recruit different scaffolding proteins that were specific to activation by insulin or BDNF. Such a phosphorylation-specific interaction has been reported for another Kv family member where Kv1.2 was associated with an actin cytoskeleton–binding protein (cortactin) and this interaction modulated channel function (Huang and Reichardt, 2003). An alternative possibility is that the total quantity of phosphorylation may be a more weighty factor than absolute position of the phosphorylation in encoding the type of modulation for Kv1.3. Activation of the epidermal growth factor, another member of the receptor tyrosine kinase family, does not cause current suppression of Kv1.3 but rather a speeding of the kinetics of channel inactivation. Interestingly, this modulation is attributable to a single phosphorylation (Y479) in the carboxyl terminus and not multiple phosphorylation (Bowlby et al., 1997).

Although both RTKs have the capacity to modulate the channel in a similar manner using an apparently similar molecular mechanism, the presence of the neurotrophin receptor appears to have a secondary effect on the channel via increasing its expression level. Production of BDNF and expression of TrkB receptor in the nervous system has been demonstrated to be activity dependent (Balkowiec and Katz, 2002). This leads to the testable hypothesis that potassium ion channel subcellular distribution or total channel expression could be altered upon upregulation of the neurotrophin signaling pathway during periods of regeneration, development, or high activity. Likewise, the downregulation of channel current could be driven both by conformational changes in the channel protein upon addition of the phosphate negative charge as well as endocytosis of the channel following BDNF stimulation. Unlike the IR signaling pathway we have studied, we know that Kv1.3 and TrkB can be co-immunoprecipitated (data not
Figure 1.6 BDNF-induced activation of TrkB receptor causes current suppression of Kv1.3 by targeting multiple tyrosine residues.  A) Cartoon depiction of the Shaker potassium channel, Kv1.3, indicating four favorable recognition motifs for tyrosine phosphorylation. A conservative Y to F single point mutation was made in each of the N and C terminal sites to eliminate the tyrosine site in the channel substrate. B) Representative current recording of a cell-attached patch from a WT Kv1.3 + TrkB transfected HEK 293 cell that was held (V_h) at -80 mV and stepped to a single depolarizing step of +40 mV (V_c) for 1000 ms. Patch pipettes were tip filled with normal patch solution and then back filled with 50 ng/ml BDNF in order to present the ligand to the external face of the membrane. The control trace (Control) is the first recording following stabilization of the patch (1-3 minutes) and the neurotrophin-treated trace (BDNF) is the same patch recorded 20 minutes later (BDNF). C-F) Same as in (B) for various Y to F channel mutants.
Table 1.2: Properties and Modulation by BDNF of Kv1.3 Current in Kv1.3 plus TrkB co-transfected HEK293 cells

<table>
<thead>
<tr>
<th>Channel Construct and Treatment</th>
<th>Peak Current (pA)</th>
<th>$\tau_{\text{inactivation}}$ (ms)</th>
<th>$\tau_{\text{deactivation}}$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Kv1.3</td>
<td>557 ± 89</td>
<td>521 ± 81</td>
<td>29 ± 7</td>
</tr>
<tr>
<td>+BDNF (n=11)</td>
<td>*477 ± 76</td>
<td>514 ± 85</td>
<td>27 ± 4</td>
</tr>
<tr>
<td>YYY111-113FFF +BDNF (n=8)</td>
<td>408 ± 77</td>
<td>899 ± 328</td>
<td>48 ± 14</td>
</tr>
<tr>
<td>Y137F Kv1.3 +BDNF (n=6)</td>
<td>394 ± 80</td>
<td>564 ± 43</td>
<td>33 ± 11</td>
</tr>
<tr>
<td>Y449F Kv1.3 +BDNF (n=8)</td>
<td>541 ± 134</td>
<td>691 ± 133</td>
<td>22 ± 3</td>
</tr>
<tr>
<td>Y479F Kv1.3 +BDNF (n=7)</td>
<td>1058 ± 159</td>
<td>561 ± 70</td>
<td>22 ± 2</td>
</tr>
<tr>
<td></td>
<td>1080 ± 185</td>
<td>598 ± 77</td>
<td>25 ± 13</td>
</tr>
</tbody>
</table>

Currents were evoked by 1-s depolarizing pulses from -90 to +40 mV in the cell-attached (c.a.) configuration. Each patch pipette was tip filled with control solution and backfilled with 50 ng/ml BDNF (see Methods and Figure 1.7). Within patch values (Mean ± S.E.M.) were taken at time 0 and 20 minute following the achievement of the c.a. configuration (+BDNF). Tau ($\tau$) values were estimated from exponential fits to the inactivating or deactivating portions of the current. * = BDNF-treated is significantly different from control, paired $t$-test, $\alpha \leq 0.05$.

Figure 1.7  Plot of time versus peak current magnitude for the experiment in Figure 1.6. Control trace represents a WT Kv1.3 transfected HEK 293 cell where the pipette was both tip and back filled with control patch solution (unstimulated with BDNF). Normalization is expressed as the initial current divided by current at the $n$th time point ($I_i / I_n$).
shown). It is possible upon activation of TrkB by BDNF, therefore, that the channel could be downregulated both by phosphorylation and by its physical removal from the membrane as TrkB receptors are recycled. Future experiments using confocal imaging combined with surface biotinylation will be required to address such questions.

While the mechanics of the modulation that occurs at discreet biochemical sites can be satisfyingly addressed using cloned proteins in a heterologous expression system, ultimately the knowledge found in these studies must be applied to native proteins to understand neuromodulation in the brain. It is important to not solely accept the most reductionist approach of a heterologous expression system as the precise mimic of the native system. Thus, it can be very useful to use both systems as parallel evidence to support a given hypothesis. Our data demonstrate that two closely-related rodent species, rat and mouse, share similar basal electrophysiological properties concerning Kv1.3-expressed currents in OB neurons. Although both from the rodentia family, it was important to establish working conditions for culturing mouse OB neurons and a description of their biophysical properties. Traditionally the smaller diameter mouse neuron was not favorably utilized as a model for studying mitral and granule single cell electrophysiology, but baseline biophysical properties are necessary if gene-targeted deletions are to be studied functionally for the olfactory bulb. Our data demonstrate that activation of either receptor tyrosine kinase, IR or TrkB, initiates Kv1.3 current suppression in both species, which may be more robust for BDNF signaling in the mouse. These data are supportive of our findings for cloned Kv1.3 expressed in HEK 293 cells, with some notable comparisons. BDNF activation of TrkB causes greater current suppression of native Kv1.3 expressed in mouse neurons over that of cloned Kv1.3 in HEK cells. The degree and type of modulation by kinases has been shown to be altered in the presence of adaptor proteins, several of which are well characterized for the neurotrophin signaling pathway. Interaction with adaptor proteins expressed in native OB neurons (Cook and Fadool, 2002) may accentuate how Kv1.3 is modulated by TrkB. Future studies will build on these reported protein-protein interactions to explore the degree and mechanism of neuromodulation in the OB using Kv1.3-null, IR kinase-null, and TrkB kinase-null mice in synchrony with studies that pinpoint the biochemical sites using heterologous expression.
CHAPTER 2

TrkB differentially regulates Shaker channels Kv1.3 and Kv1.5.

Introduction

Kv1.3 belongs to a potassium channel family that has diverse and specialized expression patterns in the developing and mature brain (Kues and Wunder, 1992; Veh R, 1995; Grosse et al., 2000). Recently, it has been discovered that the physiological role of Kv1.3 can be as diverse as setting olfactory thresholds (Fadool, 2004) to regulation of body weight (Xu et al., 2004). The biophysical properties of the voltage-gated potassium channels (Kv) can depend largely on the formation of heterotetramers with other co-expressed subunits of its Kv subfamily (Jan and Jan, 1992; Miller, 2000; Deutsch, 2002). At the cellular level, one mode of regulation of Kv channels involves kinases. Kv channels have been shown to be activated by serum- and glucocorticoid-dependent kinase (Gamper et al., 2002) in human embryonic kidney (HEK293) cells and by protein kinase C (PKC) in normal (nontransformed) human T cells (Chung et al., 2001) and retinal epithelial (RPE) cells (Strauss et al., 2002). The cell can use kinase activity in a cell-specific and stimuli-specific manner to tune electrical responses and initiate with precision a sophisticated repertoire of end points of kinase activation.

Previously we showed that brain-derived neurotrophic factor (BDNF) acting through its preferred receptor, tropomyosin-related kinase B (TrkB), can suppress Kv1.3 current in cultured mitral cells (Tucker and Fadool, 2002). We then demonstrated that Kv1.3 current is not suppressed on BDNF stimulation of co-expressed TrkB when a combination of its tyrosine residues is absent from the channel (Colley et al., 2004). We have found that many potential interacting partners of Kv1.3 in the olfactory bulb are increased in Kv1.3 -/- mice (Fadool et al., 2004). Specifically, there is more of the receptor tyrosine kinase TrkB in the olfactory bulb of
Kv1.3 +/- compared to wildtype. Kv1.3 expression also increases two fold when co-expressed with TrkB in HEK293 cells. This is interesting because it suggests that the ion channel Kv1.3, apart from exhibiting current suppression on acute BDNF activation of TrkB, may also have a relationship with TrkB such that it can directly or indirectly reciprocally regulate expression of Kv1.3 and other interacting proteins. Hence, this possibility was investigated further, the hypothesis was; with the increased expression of Kv1.3 in the plus TrkB transfection I should observe: (1) an increase in surface expression of Kv1.3, (2) an increase in Kv1.3 peak current amplitude and (3) TrkB stabilization of Kv1.3 in the cell. The next question that arose was, were these relationships unique and specific between TrkB and Kv1.3? To explore this, the effect of BDNF on Kv1.5 was measured. Kv1.3 and Kv1.5 are members of the Shaker sub-family. Secondly, was this type of interaction exclusive to TrkB and Kv1.3? The activation of another receptor tyrosine kinase, the insulin receptor (IR) kinase, is known to suppress channel activity by phosphorylation of a different and unique combination of Kv1.3 tyrosine residues compared to TrkB. Was it possible that IR could have reciprocal effects on Kv1.3? If TrkB and IR can differentially affect the expression of Kv1.3 in the olfactory bulb, then this allows TrkB and IR another level of modulation of the function of Kv1.3. The channels also exhibit differential and reciprocal modulation of the receptor tyrosine kinases, TrkB and IR, at the level of expression. The role of kinase activity in the upregulation of Kv1.3 was investigated and it was found that there was no increase of Kv1.3 expression when co-transfected with TrkB lacking the kinase. Given that the kinase activity of TrkB was necessary was it also sufficient? To answer this question, measurements were taken of the expression level of Kv1.3 tyrosine mutants when expressed in the absence and presence of TrkB. It was found, that the mere presence of tyrosine residues can impact the expression levels of Kv1.3 even in the absence of TrkB. In this study we also show that within olfactory bulb slices of mice, Kv1.3 and TrkB labeling is localized in overlapping fibers passing through the granule cell layer and on the periphery of the glomerular structures. It was also found that the TrkB receptor co-immunoprecipitates from the olfactory bulb and the cortex with Kv1.3, Kv1.5 and the insulin receptor. Hence the findings of this study have implications for the role of BDNF and TrkB modulation of Kv1.3 for processing of olfactory stimuli, information in the cortex and possibly a previously unknown interaction between Shaker channels Kv1.3 and Kv1.5, and TrkB and IR.
Materials and Methods

Solutions and Reagents. Human embryonic kidney cell (HEK 293) patch pipette solution contained (in mM): 30 KCl, 120 NaCl, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 2 CaCl₂ (pH 7.4). HEK 293 cell recording bath solution contained (in mM): 150 KCl, 10 HEPES, 1 Ethylene glycol-bis (β-amino-ethylether)-N, N, N’, N’-tetraacetic acid (EGTA), and 0.5 MgCl₂ (pH 7.4). Cell lysis buffer with protease and phosphatase inhibitors (PPI solution) contained (in mM): 25 tris (hydroxymethyl) aminomethane (pH 7.5), 150 NaCl, 150 NaF, 0.5 Ethylenediaminetetraacetic acid (EDTA), 1 sodium orthovanadate (pH 8.0), 1 phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A and 1.0% Triton X-100. Nonidet-NP40 protease and phosphatase inhibitor (NP40 PPI) solution contained the same phosphatase inhibitors as above and in mM: 20 Tris base (pH 7.5), 150 NaCl, 1 % nonidet-NP40 and 10% glycerol. Wash buffer contained (in mM): 25 Tris base (pH 7.5), and 150 NaCl, 150 NaF, 0.5 EDTA and 0.1% Triton X-100. Homogenization buffer (HB) contained in (mM): 320 sucrose, 10 Tris Base, 50 KCl, and 1 EDTA (pH 7.8). Human recombinant brain-derived neurotrophic factor (rhBDNF) was purchased from Promega (Madison, WI). Human recombinant insulin was purchased from Roche (Indianapolis, IN). Cycloheximide was purchased from Calbiochem (San Diego, CA). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Fair Lawn, NJ). Tissue culture reagents were purchased from Gibco/BRL (Gaithersburg, MD). All salts were purchased from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Houston, TX). In biochemistry experiments transfected cells were stimulated with 100 ng/ml BDNF or 10 ng/ml insulin.

cDNA Constructs and Antibodies. Kv1.3 channel was subcloned into the multiple cloning region of pcDNA3 (Invitrogen) using the unique restriction site HindIII. Human Kv1.5 channel tagged at the C-terminal end with the C-peptide antigen and cloned into pCS2 was a gift from Dr. Todd C. Holmes, New York University (Nitabach et al., 2001; Nitabach et al., 2002). IR cDNA was a gift from Dr. R. Roth, Stanford University in the pECE vector and was subcloned into the multiple cloning region of pcDNA3 (Invitrogen) using the unique restriction site SalI and XbaI. TrkB cDNA was a generous gift from Dr. P. Barker, McGill University in a CMX vector. All channel and kinase coding regions were downstream from a cytomegalovirus.
(CMV) promoter. The N-terminus of the α-subunit of Kv1.3 fused to the green fluorescent protein (GFP) in pEGFP-C1 was a generous gift of Dr. J. Kupper, Max-Planck Institute (Kupper, 1998). The 10-amino acid c-myc epitope (EQKLISEEDL) was inserted into the S1/S2 loop between residues 226-227 of Kv1.3 channel via two consecutive PCR reactions using Expand Long Template PCR System, Roche (Indianapolis, IN).

AU13, a rabbit polyclonal antiserum, was generated against a 46 amino acid sequence (MVIEEGGMNHSAFPQTPFKTNSTACTTTNNPPNDCVNIKKIFTDV)523 representing the unique coding region of Kv1.3 on the C-terminus. Genmed Synthesis (San Francisco, CA) purified the peptide and Cocalico Biologicals (Reamstown, PA) the antisera as previously characterized in Tucker and Fadool (Tucker and Fadool, 2002). This antibody was used for immunoprecipitation (1:1000), Western blot detection (1:5000) of Kv1.3 and immunohistochemistry (1:1000). Tyrosine phosphorylated proteins were visualized by the anti-phosphotyrosine antibody 4G10 from Upstate Biotechnology (Lake Placid, NY) and used at 1:1000 for Western blots. Tyrosine phosphorylated proteins were immunoprecipitated with 4G10 antibody (3µg per 600-1200 µg of lysate). Monoclonal antiserum directed against amino acids 156-322 of human TrkB was purchased from Transduction Laboratories (San Diego, CA) and used at 1:1000 for Western blots and 1:500 for immunohistochemistry. Rabbit polyclonal Insulin Receptor β antibody was purchased from BD Biosciences Pharmingen (San Jose, CA) and used for Western blots at 1:1000. Rabbit anti-Kv1.5 purified polyclonal antibody against an intracellular, C-terminal portion of mouse Kv1.5 protein (amino acids 513-602) was purchased from Chemicon International (Temecula, CA) and used for Western blots at 1:1000. GRB10 (K-20) rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. Monoclonal Anti-β actin antibody was purchased from Sigma (St. Louis, MO) and used at 1:750 for Western blots or rabbit polyclonal Anti-β actin antibody from Sigma (A 2066) used at 1:1000. Anti-c-myc mouse monoclonal antibody (clone E910) to a peptide (EQKLISEEDL) from the human myc protein was purchased from Roche (Nutley, NJ) and used for Western blots. Goat anti-rabbit FITC-conjugate (1:100) antibody and Texas Red goat anti-mouse (1:200) was from Molecular Probes.

**HEK 293 Cell Culture and Transient Transfection.** HEK 293 cells were maintained in Minimum Essential Medium (MEM), 2% penicillin/streptomycin, and 10% FBS (Gibco
Cells were grown to 95% confluency dissociated with trypsin-EDTA (Sigma) and mechanical trituration, diluted in MEM to a concentration of around 600 cells/ml, and plated on Corning dishes (Catalog # 25000, Fisher Scientific). Before transfection, cells were grown to between 50-60% and 80-95% confluency for electrophysiology or biochemistry respectively. cDNA was introduced into HEK 293 cells with a Lipofectamine™ Transfection Reagent, Invitrogen (Carlsbad, CA) 3-5 days after passage as previously described (Fadool et al., 1997). Briefly, cells were transfected for 4-5 hrs using 2.0 µg of each cDNA construct per 60 mm dish for biochemistry or 4-5 hrs using 1.0 µg of each cDNA construct per 35 mm dish for physiology. Cells were then harvested for biochemistry or used for patch-clamp recording approximately 24-48 hrs post transfection.

Cycloheximide-chase assay experiments fashioned after Cullen et al. experiments (Cullen et al., 2004) were used to determine the half-life of Kv1.3, alone versus with TrkB. Cycloheximide was used at a final concentration of 30 µg/ml and 100 µg/ml and added to dishes 20-24 hours post-transfection. Cells were then harvested at 0, 2, 4, 6 and 8 hours after addition of cycloheximide. Bradford assay was carried out and protein concentration was determined by the average of readings obtained for duplicates of each sample. DMSO was used at a final concentration of 0.03%, as the vehicle control condition to determine that there was no significant difference between protein expression in the absence or presence of DMSO. Experiments were repeated a minimum of three times.

Electrophysiology. Hoffman modulation contrast optics was used to visualize HEK 293 cells at 40X magnification (Axiovert 135, Carl Zeiss). Macroscopic currents in cell-attached membrane patches were detected using an Axopatch-200B amplifier (Axon Instruments). All electrophysiological data were recorded and analyzed using pClamp 8.0 and 9.0 software in combination with the analysis package of Origin, MicroCal Software (Northampton, MA) and Quattro Pro, Borland International (Scotts Valley, CA). Patch electrodes were fabricated from Jencons glass (Jencons Limited, Bedfordshire, UK), fire-polished to ~1 µm, and coated near the tip with beeswax to reduce the electrode capacitance. Pipette resistances were between 9 and 14 MΩ. Currents were recorded 24-48 hrs after transfection. The diameter of the patch pipette, and hence number of ion channels sampled, was held uniform by checking the bubble number of the pipette immediately after electrode fabrication and polishing (Mittman et al., 1987). In general,
currents were recorded from cell-attached patches held at a holding potential \((V_h)\) of \(-90\) mV and stepped to \(+40\) mV depolarizing potentials \((V_c)\) with a pulse duration of 1000 ms for Kv1.3 and 10 ms or 200 ms for Kv1.5. Pulses were delivered at 45-s intervals in order to prevent cumulative inactivation of Kv1.3 channels. The same 45-s interpulse interval was delivered for Kv1.5 to allow comparison with Kv1.3. Peak outward current amplitude, time constants of channel inactivation and deactivation were determined by parameter fitting of traces exhibiting stability around the 3rd to 5th minute into the recording. The inactivation time constant was determined by fitting the current trace activated by a \(-90\) mV to \(+40\) mV depolarizing step with a two-term exponential standard function (using Chebyshev’s technique in Clampfit 8.0 and 9.0 software) according to the following equation:

\[
I(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) + C
\]

where \(A_1\) and \(A_2\) are the amplitudes, \(\tau_1\) and \(\tau_2\) are the time constants and \(C\) is the offset constant. The inactivation time constant was calculated as the weighted average of the two time constants. Deactivation time constant was determined by the fit to one-term using the above exponential standard function to the tail current. BDNF stimulation was accomplished by tip-filling (~0.01 mm) the patch-pipette with control solution and then back filling (~35 mm) with BDNF (1 ng/µl). The dilution of the BDNF using this procedure would be insignificant due to the ratio of the volume (back-fill/tip-fill) difference that estimated to be at least 1 million.

**Immunocytochemistry.** Twenty-four to 48 hours after transfection, cells were fixed with 1% paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes at room temperature. This was followed by three 10-minute washes with PBS. Afterwards, aspecific protein binding was blocked with 30 minutes incubation in 2% bovine serum albumin (BSA) in PBS. Surface c-myc-tagged channels were labeled at room temperature for 90 minutes with mouse c-myc antibody at 1:800 dilution in 2% BSA in PBS. This was followed by three 10-minute washes in PBS and the permeabilization in 2% BSA in 0.1% Triton X-100 and PBS (PBST) for 45 minutes. Total channel was labeled using rabbit anti-Kv1.3 antibody (anti-AU13) in 2% BSA in PBST, followed by three 10-minute washes in PBST. Subsequent incubation with fluorescent secondary antibodies, Alexa Fluor 488-conjugated goat anti-rabbit (1:200) and Texas Red-conjugated goat anti-mouse (1:100) was performed in dark for 90 minutes. This was followed by a wash with PBST, five minutes incubation with diamidino-phenyindole (DAPI) in
PBS (1:5000) and a final wash in PBS. Coverslips were mounted onto slides with 70/30% glycerol/PBS, containing p-phenylene-diamine. For imaging distribution of the c-myc-tagged channel, permeabilization step and labeling with anti-Kv1.3 and anti-rabbit secondary antibody were omitted, and all reagents were dissolved in PBS instead of PBST.

**Immunohistochemistry.** Mice postnatal day 20-30, were perfused with paraformaldehyde and 12 µm sections were made on a cryostat and mounted onto 2% gelatin coated slides. Sections were stored at −20°C until used. Sections were air-dried for 30mins and rinsed 3 times with 1x PBS (Ph 7.4) every 5 minutes. Sections were then incubated for 30 minutes in blocking solution made with 10 % Goat Serum in PBS containing 0.3% Triton-X-100. The blocking solution was then drained and any excess blotted off the slide. Primary antibody was diluted in blocking solution and applied to the sections for 2 hours at room temperature or overnight at 4°C. The sections were then rinsed 3 times in PBS every 5 minutes and the secondary antibody applied in blocking solution as above for 2 hours at room temperature or overnight at 4°C. The sections were handled in the dark from hereon. The double labeling experiments were performed by repeating one more round of labeling using another primary against the second protein to show co-localization. The sections were then given a final rinse in PBS for 5 minutes followed by a 5 minute rinse in distilled water and mounted using Vectasheild (Vector Laboratories, CA). The edges of the cover slip were sealed and slides were stored at 4°C until imaging. Control slides were treated exactly the same except the primary antibodies were omitted. Sequential labeling was done using AU13 as the first primary antibody and labeled with FITC-conjugated donkey anti-rabbit followed by anti-TrkB labeled with Texas Red-conjugated goat anti-mouse.

**Confocal Microscopy and Image Analysis.** Microscopic analysis of fluorescently labeled HEK 293 cells was performed on a Zeiss Axioplan 2 Microscope attached to a Zeiss LSM510 confocal system (Carl Zeiss, Germany). Samples were excited at 488 nm (for GFP) and 595 nm (for Texas Red) through a 63x oil-immersion objective (NA=1.40) and fluorescence between 500-545 and 565-615 nm was detected from <0.8 µm thick sections. Images were taken from the cells at their maximal cross section. Cell outlines were identified using the Texas Red labeling. Green channels of the images were analyzed using Metamorph software (Universal Imaging). After removing background pixels by thresholding, integrated pixel intensity was
calculated both from the total cross section of the cell and from an area that encompassed the cross section of the cell excluding the outermost region.

**Immunoprecipitation and Electrophoretic Separation.** For immunoprecipitation, harvested OB or cortex was homogenized using a Kontes tissue grinder (size 20; 50 strokes) in ice-cold HB containing 0.5% Nonidet P-40 detergent in PPI solution (see “Solutions and Reagents”). Lysis was continued on a Roto-Torque slow speed rotary (model 7637; Cole Palmer, Vernon Hills, IL) for 10 minutes at 4°C. The lysates were clarified by centrifugation at 14,000 X g for 10 minutes at 4°C and precleared for 1 hour with 3 mg/ml protein A-sepharose (Amersham-Pharmacia, Newark, NJ), which was followed by another centrifugation step to remove the protein A-sepharose. Protein concentration was determined in triplicates by Bradford assay. Lysates were obtained from transfected HEK 293 cells as previously described (Colley et al., 2004). Kv1.3 or Kv1.5 channel proteins, or TrkB and IRβ receptors were immunoprecipitated from native tissue or transfected HEK 293 cells. Clarified lysates were incubated overnight at 4°C with 3 µg of respective antisera or antibody per 600-1200 µg of HEK 293 cell lysate or 2000-3000 µg OB tissue lysate. Immunoprecipitates were then collected by 2 hours incubation with protein A-sepharose and centrifugation as above and washed 4 times with ice-cold NP40 PPI solution for native tissue or with ice-cold wash buffer for HEK 293 cells. Lysates and washed immunoprecipitates were diluted 1:1 in sodium dodecyl sulfate (SDS) gel loading buffer (Sambrook et al., 1989) containing 1 mM Na₃VO₄ and stored at –20°C for subsequent analysis. Immunoprecipitated proteins from native tissue or transfected HEK 293 cells were separated on 10% acrylamide gels by SDS-PAGE and electro-transferred to nitrocellulose blots. Blots were blocked with 5% nonfat milk and incubated overnight at 4°C in primary antisera against either Kv1.3, Kv1.5, TrkB, IRβ or in 4G10 antibody. They were then incubated with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit secondary antibody (1:6000) (Amersham-Pharmacia) or HRP-conjugated goat anti-mouse secondary antibody (1:6000) (Sigma) for 60 minutes at room temperature. Enhanced chemiluminescence (ECL; Amersham-Pharmacia) exposure on Fugi Rx film was used to visualize labeled proteins. The film autoradiographs were analyzed by quantitative densitometry using a Hewlett-Packard PhotoSmart Scanner (model 106-816, Hewlett Packard, San Diego, CA) in conjunction with Quantiscan software (Biosoft, Cambridge, England). The raw densitometry values for the
different groups were obtained from the same autoradiograph and therefore same exposure time for each batch of experiments and then analyzed using a Student’s \( t \)-test.

**Results**

**Kv1.3 expression is increased in the presence of TrkB and is kinase dependent.** Kv1.3 co-expressed with TrkB shows an increase in expression which showed a two fold increase. The two fold increase was significantly different from Kv1.3 alone conditions in Human Embryonic Kidney (HEK 293) cells (Figure 2.1A). The increase ranged on average between 1.8 to 2.3 times and was analysed from a total of 18 transfections. Of these 18 transfections, some were done on different days and using HEK 293 cells plated from different cell passages (2.1 ± 0.2, \( n=18 \)). We then asked whether the kinase activity of the TrkB receptor might be important for TrkB regulation of Kv1.3 expression and found that Kv1.3 co-transfected with TrkB lacking the kinase did not show an increase in expression (0.8 ± 0.2, \( n=4 \)) see Figure 2.1B. These data suggest that the kinase activity in the cell is an important aspect of Kv1.3 upregulation. Note that the cells were grown in normal culture media containing fetal bovine serum which will contain the neurotrophic factors such as BDNF and NT-4 both which bind and activate TrkB albeit BDNF with the highest affinity.

Figure 2.2 A shows how expression of 1.5 µg of Kv1.3 increases with TrkB cDNA quantity and plateaus when the cDNA quantities are approaching a 1:1 ratio of Kv1.3:TrkB. Figure 2.2 B shows expression changes of Kv1.3 when increasing µg concentrations of Kv1.3 cDNA are co-transfected with 2.0 µg of TrkB cDNA. The maximum expression level for Kv1.3 in 10 µg of whole cell lysate seems to plateau around the same 1:1 ratio of Kv1.3:TrkB as seen in Figure 2.2 A.

*Shaker channels Kv1.3 and Kv1.5 differential regulation of current and protein expression by receptor tyrosine kinases, TrkB and IR.* The next step investigated if the above finding represented a unique and specific interaction between TrkB and Kv1.3. To explore this, the experiments were repeated using a closely related ion channel, *Shaker* sub-family member Kv1.5. In contrast to Kv1.3, a 50% decrease in the expression of Kv1.5 was observed (Figure 2.4 A, B). This decrease was not matched by a decrease in Kv1.5 current for Kv1.5 + TrkB
condition (Figure 2.4 C, D). However, Kv1.3 currents were increased compared to recordings from cells of the Kv1.3 alone condition. When the insulin receptor (IR) was co-expressed with Kv1.3, there was a decrease in the expression of Kv1.3 (Figure 2.3 A, B) but no increase in current (Figure 2.3 C, D). IR was also co-expressed with Kv1.5 but there was no discernible effect on the expression of Kv1.5 or on Kv1.5 current (Figure 2.4 C, D). TrkB up-regulates Kv1.3 expression but down-regulates Kv1.5 expression. IR on the other hand down-regulates Kv1.3 expression but has no effect on Kv1.5 expression. Interestingly, both Kv1.3 and Kv1.5 down-regulate the expression of both TrkB and IR (data not shown).

Figure 2.1 Kv1.3 expression levels when co-transfected with TrkB or TrkB lacking kinase activity. HEK 293 cells were transiently transfected with Kv1.3 (K) cDNA minus or plus TrkB (KT) or TrkB lacking its kinase activity, D for dead kinase (KD). The cells were lysed and cellular debris spun down and removed then the protein concentration determined by Bradford
Assay done in triplicate. Ten µg of whole cell lysates from each transfection condition was separated by SDS-PAGE, electro-transferred to nitrocellulose and probed with anti-Kv1.3. A, upper panel, shows representative expression bands for Kv1.3 alone versus Kv1.3 and TrkB, the result from three experiments. A, lower panel, shows Kv1.3 versus Kv1.3 plus TrkB and Kv1.3 plus TrkB lacking its kinase domain. B, Histogram plot of the normalized immunodensity values for repeats of experiments shown in A and B above. Immunodensity values for K vs KT and K vs KD were compared using Student’s t test, * significantly different from K channel at α ≤ 0.05.

GFP N-terminal tagged Kv1.3 channel expression is not increased in the presence of TrkB. In an attempt to observe any membrane versus cellular distribution pattern changes that may have accompanied the increase in Kv1.3, surface and intracellular tagged Kv1.3 channels were constructed. A myc epitope (inserted into the S1/S2 loop) tagged Kv1.3 channel and one containing both a myc and N-terminal GFP double-tagged channel were made as shown in the cartoon representation in Figure 2.5 A. The Kv1.3 channel containing the N-terminal GFP tag did not exhibit increased expression in the presence of TrkB even though the myc-tagged channel expression was increased. The result of co-expression of myc-tagged Kv1.3, mycGFPKv1.3 and GFPKv1.3 -/+ TrkB is shown in Figure 2.5. The peak outward current from cells transfected with tagged-Kv1.3 -/+ TrkB is shown in C, where an increase in channel current is observed in cell-attached membrane patches in the KT condition for the mycKv1.3 but not for the GFPKv1.3 or the mycGFPKv1.3. Figure 2.7 demonstrates there is also not increase for surface gfpMycKv1.3 in the presence of TrkB while surface MycKv1.3 increases the presence of TrkB, these reflect the results obtained for current and protein expression in the same conditions.

TrkB kinase increases the stability of cellular pools of Kv1.3. Exactly how TrkB was able to increase the expression of Kv1.3 was the next question. In these transiently transfected HEK293 cells it was possible that the increased expression of Kv1.3 cDNA resulted from increased translation or expression rates. It could also result from increased cellular stability of the Kv1.3, in effect slower rates of degradation. Cycloheximide is a protein synthesis inhibitor. To test whether TrkB might be increasing the stability of Kv1.3 in the cell we carried out a Cycloheximide chase study as per Cullen et al. (2004). If TrkB increases the stability of Kv1.3 in the cell then we expected to see a steady decline in the amount of Kv1.3 over time after exposure to cycloheximide in the Kv1.3 alone condition but not in the Kv1.3 + TrkB condition.
Kv1.3 +/- TrkB transfected cells were treated with cycloheximide (30 μg/ml and 100 μg/ml) for 0, 2, 4, 6 and 8 hours and the cells harvested at these time points and Kv1.3 levels in 10 μg quantities of lysate measured by Western Blot analysis as shown in Figure 2.9 A.

Figure 2.2 Expression levels of Kv1.3 with various ratio concentrations of TrkB:Kv1.3 cDNA co-transfection. HEK 293 cells were transiently transfected with Kv1.3 (K) cDNA minus or plus TrkB (+T). The cells were lysed and cellular debris spun down and removed then the protein concentration determined by Bradford Assay done in triplicate. 10 μg of whole cell lysates from each transfection condition was separated by SDS-PAGE, electro-transferred to nitrocellulose and probed with anti-Kv1.3. A, Representative expression bands for 1.5 μg of Kv1.3 cDNA alone compared with increasing quantities of TrkB cDNA. B, Representative expression of Kv1.3 observed for varying μg values of Kv1.3 cDNA versus the same varying concentrations of Kv1.3 co-transfected with 2.0 μg of TrkB cDNA.

The experiments were also carried out at 0, 3, 6, 9 and 12 hours with similar results (data not shown). The results of three such experiments were quantified using densitometry analysis and the half-life of Kv1.3 increased from 3.8 ± 1.4 (mean ± S.E.M) hours in the presence of TrkB to
The mean half-life of Kv1.3 was 1.7 times greater in the presence of TrkB and increased on average 2.5 ± 1.1 (n=3).

Figure 2.3 Differential effects of co-expression of TrkB and IR on Kv1.3 expression and current. HEK 293 cells were transiently transfected with Kv1.3 (K) cDNA minus or plus TrkB (KT) or IR (KI). A, The cells were lysed and cellular debris spun down and removed then the protein concentration determined by Bradford Assay done in triplicate. Ten μg of whole cell lysates from each transfection condition was separated by SDS-PAGE, electro-transferred to nitrocellulose, probed with anti-Kv1.3 and blotted with anti-actin to double check transfection efficiency. B, Histogram of normalized densitometry values of K vs KT and KI conditions. C, Representative Kv1.3 current from cell-attached recordings of conditions in A. Cell-attached membrane patches were held at −90 mV and stepped from −80 to +40 mV in 10 mV increments for a duration of 400 ms using 30 s interpulse intervals between voltage steps. C, Histogram of peak current recordings (Mean ± S.E.M.) as shown in B. Immunodensity values for K vs KT and K vs KI were compared using Student’s t test, * significantly different from K at α ≤ 0.05. D, Histogram representing mean peak current (pA) ± S.E.M. from cell-attached recordings of conditions shown in A.
Figure 2.4 Differential effects of co-expression of TrkB and IR on Kv1.5 expression and current. HEK 293 cells were transiently transfected with Kv1.5 (K) cDNA minus or plus TrkB (KT) or IR (KI). A, Same as Figure 2.3 A but for Kv1.5. B, Histogram of normalized densitometry values of K vs KT and KI conditions. C, Representative Kv1.3 current from cell attached recordings of conditions in A. Cell-attached membranes were held at –90mV and stepped from –80 to +40 mV in 10 mV increments for a duration of 400 ms using 10s interpulse intervals between voltage steps. D, histogram of peak current recordings (Mean ± S.E.M.) as shown in B. Immunodensity values for K vs KT and K vs KI were compared using Student’s t test, * significantly different from K at $\alpha \leq 0.05$. 
Figure 2.5 Effects of GFP-N terminal versus extracellular myc surface Kv1.3 tag on TrkB induced increase of Kv1.3 expression and current. A, cartoon showing position along channel structure of N-terminal GFP tag and myc surface tag inserted on the extracellular loop between transmembrane domain 1 and 2 of Kv1.3. HEK 293 cells were transiently transfected with myc tagged Kv1.3 (Myc), GFP tagged Kv1.3 (GFP) or double tagged Kv1.3 (gfpMyc) cDNA minus (K) or plus TrkB cDNA (KT). B, the cells were lysed and cellular debris spun down and removed then the protein concentration determined by Bradford Assay done in triplicate. Ten µg of whole cell lysates from each transfection condition was separated by SDS-PAGE, electrotransferred to nitrocellulose and probed with anti-Kv1.3. The gfp and gfpMyc tagged Kv1.3 band runs higher due to the added weight of the gfp portion of the tagged channel. C, representative current recordings of cell-attached patches from K vs KT, for Kv1.3 tagged with only Myc (top panel), tagged with gfp and Myc (middle panel) and gfp alone (bottom panel). Cell-attached patches were held at −90 mV and stepped to a single depolarizing step of +40 mV for 1000 ms. D, Histogram of mean peak current ± S.E.M. for the recordings as shown in C. E, Histogram of densitometry analysis of Kv1.3 expression in B. Mean peak current values for K
vs KT (for Myc, gfpMyc and gfp) were compared using Student’s t test, * KT significantly different from K at $\alpha \leq 0.05$.

Figure 2.6 Cycloheximide inhibition of protein synthesis on Kv1.3 expression in the presence and absence of TrkB. HEK 293 cells were transiently transfected with Kv1.3 (K) cDNA minus or plus TrkB (KT) and protein expression was chased in the presence of 100 $\mu$g/ml Cycloheximide for 0, 2, 4, 6 and 8 hours and 30 $\mu$g/ml Cycloheximide for 0, 3, 6, 9 and 12 hours. A, The cells were lysed at the various time points, cellular debris spun down and removed and then the protein concentration determined by Bradford Assay done in triplicate. Ten $\mu$g of whole cell lysates from each transfection condition was separated by SDS-PAGE, electro-transferred to nitrocellulose and probed with anti-Kv1.3. A, Representative result obtained for 100 $\mu$g/ml cycloheximide chase Kv1.3 expression assay (top panel) and 30 $\mu$g/ml cycloheximide (bottom panel). B, Scatter plot of the mean densitometry values ± S.E.M. for three experiments using 100 $\mu$g/ml cycloheximide as shown in B. Densitometry pixel density, in each experiment, was normalized to the 0 hour time point for the K and the KT conditions.

Tyrosine residues important for regulation of Kv1.3 expression. Tyrosine residues on Kv1.3 can be phosphorylated by tyrosine kinases and in Figure 2.8 A the blots demonstrate that
both TrkB and IR presence results in an increase in the tyrosine phosphorylated state of the channel. If TrkB was increasing the stability of Kv1.3 in the cell but Kv1.3 expression was not increased in the presence of TrkB lacking the kinase we asked whether the phosphorylation of Kv1.3 tyrosine residues was necessary for the increase and whether the channel had to also be functional. To investigate the importance of Kv1.3 tyrosine residues we utilized Kv1.3 tyrosine mutants containing phenylalanines (F) instead of tyrosines (Y) at specific known tyrosine phosphorylation target sites: 111-113, 137, 449 and 479. To investigate the importance of function we used W386F, a Kv1.3 channel containing a mutation in the pore, a tryptophan (W) residue was mutated to a phenyalanine and this prevents the pore from conducting K\(^+\) ions. Kv1.3, the various mutants and W396F were transfected -/+ TrkB and 10 \(\mu\)g of lysate from each condition was run in a lane and blot with anti-Kv1.3. A representative blot of the results of four experiments is shown and quantified in a histogram in Figure 2.8 B and C respectively. In the absence of any combination of tyrosine residues and of a fully functional channel there is no significant increase in Kv1.3 protein. However, analysis of the relative expression of Kv1.3 for each condition to the WT Kv1.3 as graphed in Figure 2.8 D, indicates that YYY111-113FFF mutant expresses significantly less protein than WT Kv1.3 while W386F non-conducting Kv1.3 expresses significantly more channel than WT Kv1.3 by Students’s \(t\) test on densitometry values.

**Kv1.3 and TrkB co-localize in the olfactory bulb.** Our observation of increased Kv1.3 in the presence of TrkB led us to investigate the relationship between Kv1.3 and TrkB, however exactly where Kv1.3 and TrkB interact in the olfactory bulb is not known other than that they were present in the soma of mitral cells in culture (Fadool and Levitan, 1998). Although the initial studies showing BDNF modulation of Kv1.3 in olfactory bulb neurons was recorded from primary neurons in culture (Tucker and Fadool, 2002), the distribution of Kv1.3 is likely not going to be confined to the soma of neurons in vivo. The larger objective to understanding the relationship between Kv1.3 and TrkB is to finally be able to put their relationship into the context of the brain, to unravel the role of this modulation and how it contributes to olfactory bulb function. The olfactory bulb does not function in isolation but works in tandem with circuits in the cortex to give meaning to our odor world. To ascertain that the relationships between the *Shaker* channels Kv1.3 and Kv1.5 and tyrosine kinases TrkB and IR are relevant to the olfactory bulb and cortex, co-immunoprecipitation studies were carried out.
Figure 2.7. Surface expression of MycKv1.3 and gfpMycKv1.3 in the presence of TrkB. HEK 293 cells were transfected using the MycKv1.3 and gfpMycKv1.3 tagged channels as in Figure 2.5 in the absence and presence of TrkB. Twenty-four to 48 hours after transfection, cells were fixed and MycKv1.3 was labelled with anti-myc in non-permeabilization conditions, permeabilized, then total channel labelled with anti-Kv13 followed by the secondary antibodies, then incubation with a nuclear stain, diamidino-phenyindole (DAPI). A, representative images of MycKv1.3 surface labelling in the -/+ TrkB condition. B, histogram of the plasma membrane ratio of surface MycKv1.3 pixel values/total MycKv1.3 in the cell pixel values. * Statistically significant difference from Student’s t test of the surface to total pixel densities of K vs KT for MycKv1.3, α ≤ 0.05. C, histogram of plasma membrane ratio of gfpMycKv1.3 surface to total pixel densities of K vs KT for gfpMycKv1.3.
Figure 2.8 Kv1.3 phosphorylation by TrkB and IR kinases and expression levels of Kv1.3 mutants in the presence of TrkB. HEK293 cells were transfected with Kv1.3 wild-type (WT) or Kv1.3 mutants and TrkB or IR cDNA. The cells were lysed, cellular debris spun down and removed and then the protein concentration determined by Bradford Assay done in triplicate. 10 μg of whole cell lysates from each transfection condition was separated by SDS-PAGE, electrotransferred to nitrocellulose and probed with anti-Kv1.3 antibody. To measure phosphorylation levels of Kv1.3, Kv1.3 was immunoprecipitated from equal weights of lysate for the different conditions. A, Kv1.3 was co-transfected with -/+ TrkB or -/+ IR and stimulated -/+ BDNF (100ng/ml) or -/+ insulin (10 ng/ml) respectively. Lysates were blotted with anti-Kv1.3 and immunoprecipitated Kv1.3 was blotted with anti-phosphorylated tyrosine antibody (anti-PY). B, Kv1.3 WT, tyrosine mutants (YYY111-113FFF, Y137F, Y449F and Y479F) and a non-conducting pore mutant (W386F) were transfected -/+ TrkB and 10 μg whole cell lysates from these conditions blotted with anti-Kv1.3. C, Histogram representing normalized densitometry analysis of Kv1.3 experiment in B for WT Kv1.3, mutants and W386F non-conducting Kv1.3 plotted as a ratio of Kv1.3 in the +TrkB/-TrkB conditions. D, Histogram of Kv1.3 expression as a ratio of Kv1.3 expression levels in the mutant Kv1.3 conditions to the WT.
Kv1.3. Pair-wise comparison of log of the densitometry values of K vs KT and Mut/WT using Student’s $t$ test, *significantly different at $\alpha \leq 0.05$.

To define where Kv1.3 and TrkB may possibly interact in olfactory bulb circuitry, co-localization studies were done using C57 mice (postnatal day 20-30) olfactory bulb slices. This is the first study of its kind in the olfactory bulb. In Figure 2.9 Kv1.3, Kv1.5, TrkB and IR were immunoprecipitated from equal $\mu$g weights of olfactory bulb and cortex homogenates taken from C57 mice. In the top blots TrkB bands were obtained from immunoprecipitates of Kv1.3 and Kv1.5, with less of the 145 kDa form of TrkB compared to identical IP protocols done in the cortex blots below. In both olfactory bulb and cortex the 95 kDa form of TrkB lacking the kinase seems to co-immunoprecipitate in greater densities with Kv1.3, Kv1.5, TrkB and IR compared to the 145 kDa form. Kv1.3 in the cortex seems to associate more with 145 kDa TrkB than in the olfactory bulb. In Figure 2.9 II, Kv1.3 labeling in the green channel is evident starting from the exterior circumference of the olfactory bulb in fibers of the glomerular layer (GL), external plexiform layer (EPL), mitral cell layer (MCL), internal plexiform layer (IPL) and the granule cell layer (GCL), no signal is observed in the outer nerve layer. TrkB labeling is seen in the red channel in III (A, D, G) and Kv1.3 in the green channel in III (B, G, H). Kv1.3 and TrkB distribution patterns overlap mainly in fibers passing through the internal plexiform layer (III, C), slightly in some fibers surrounding the glomeruli in a closer magnification of the GL (III, F) and in the granule cell layer (III, I).

**Discussion**

The main finding in this paper is that the TrkB receptor can regulate the expression of Kv1.3 and it appears to do so by stabilizing the protein in the cell. The kinase domain of TrkB is necessary for TrkB to increase the expression of Kv1.3 but not sufficient as it also requires the presence of specific tyrosine residues in Kv1.3. Specifically the Kv1.3 YYY111-113FFF mutant expresses at levels 50% lower than the WT but TrkB co-expression did not restore the expression level to that of WT alone or the equivalent of a two fold increase. The other tyrosine Kv1.3 tyrosine mutants Y137F, Y449F and Y479F also did not exhibit an increase in expression. This lends
Figure 2.9 TrkB association with Shaker channels and IR kinase in the cortex and olfactory bulb. I, olfactory bulb and cortex were harvested from C57 mice (postnatal day 20-30), homogenized and lysed. Lysates were clarified by centrifugation and from the supernatants any native immunoglobulins were removed by pre-clearing with protein A-sepharose. The protein
concentration was determined by the average of triplicate samples analyzed by Bradford assay. Equal weights of lysate (2000-3000 μg) were incubated overnight with Kv1.3, Kv1.5, TrkB and IR antibody (3 μg of antibody per 600-1200 μg of lysate) and then incubated for 2 hours with protein A-sepharose. The immunoprecipitated proteins (IP) were collected after the final rinse and centrifugation step by retaining the pellet and dissolving it in SDS gel loading buffer. In each lane 25-30 μg of the IPs were separated by SDS-PAGE, electro-transfered to nitrocellulose and blotted with anti-TrkB antibody. II and III, C57 mice were anaesthetized at postnatal day 20-30 and perfused. Olfactory bulbs were dissected, post-fixed, cryoprotected, sectioned on a cryostat at 10 μm in the coronal plane and mounted on 2% gelatin coated slides. Sections were labeled with Kv1.3 antibody (AU13) in II. In III sections were labeled with AU13 (1:1000) labeled with secondary antibody for the green channel followed by labeling with a second antibody and then labeled with a second antibody against that second antibody for the red channel (III). The control sections were treated exactly the same except the primary antibodies were excluded (II, D). II, Kv1.3 labeling in the olfactory bulb layers is seen in the green channel (A, B, C). The merger of the two shows overlapping regions as yellow in III (C, F, I). Starting from the outer circumference of the olfactory bulb: olfactory nerve layer (ONL), glomerular layer (GL), external plexiform layer (EPL), mitral cell layer (MCL), internal plexiform layer (IPL), granule cell layer (GCL).

credence to the idea that the presence of tyrosines may be important as a signal to the cell. It might signify that the channel is functional and contains sites that can be covalently modified by addition of phosphates thereby allowing for specific modulation of biophysical properties as the need arises. The importance of tyrosine residues at any discrete site in K channels cannot be underestimated. In a very convincing set of experiments Henry Lester and his group showed that the tyrosine residue at position 242 of the inward rectifier Kir2.1, when decaged, led both to decreased K+ currents and also to substantial decreases in membrane capacitance in *Xenopus* oocytes (Tong et al., 2001). They also showed that decaging of this single tyrosine residue led to massive clathrin-mediated endocytosis with many clathrin-coated vesicles being withdrawn from the oocyte surface for each Kir2.1 channel inhibited. Therefore phosphorylation of this residue or as they described it: “the changing of the structure of a single residue in a single species of membrane molecule in effect results in the molecule being removed from the membrane by massive membrane trafficking”. The membrane trafficking was calculated to be 15-26 % in 30 minutes. The Kv1.3 non-conducting pore mutant (W386F) did not increase in expression in the presence of TrkB. W386F expression levels started at much higher levels than WT Kv1.3 per 10 μg of lysate. TrkB also differentially modulates Kv1.5 expression, it increases Kv1.3 expression but decreases the expression of Kv1.5. It is perhaps worth investigating if this opposite effect on
the expression of Kv1.3 and Kv1.5 can occur in microglia via TrkB as there was a reported ‘switch’ from Kv1.5 to Kv1.3 current when microglia become activated (Kotecha and Schlichter, 1999). The differential regulation of ion channels by neurotrophins is not uncommon, it has been observed that NGF and BDNF in human neuroblastoma NB69 cells produce the expression of a fast tetrodotoxin-sensitive Na\(^+\) current after day 3 but suppressed the slow tetrodotoxin-resistant variety (Urbano and Buno, 2000). In differentiation the regulation of channel protein expression is crucial for survival and formation of circuits and it has been postulated that BDNF and TrkB may serve to help differentiation and survival of neuronal subtypes in the olfactory bulb (Schwob et al., 1992; Deckner et al., 1993; Buckland and Cunningham, 1998). Neuronal depolarization was found to control BDNF-induced upregulation of NR2C NMDA receptor mRNA via the TrkB-extracellular signal-regulated kinase (ERK) 1/2 cascade (Suzuki et al., 2005). The importance of the BDNF-TrkB pathway was supported convincingly by the significant reduction in NR2C in normally migrated granule cells of TrkB\(^{-/-}\) knock-out mice in vivo.

On the other hand the insulin receptor (IR) seems to suppress the expression of Kv1.3. This finding confirms that IR can modulate Kv1.3. Kv1.3 has recently been implicated in the way that insulin acts in the body to remove excess glucose from the blood by translocating GLUT4 (Li et al., 2006) to the membrane and regulating peripheral insulin sensitivity (Xu et al., 2004). Another relevant finding worth considering is that a new variant of the human Kv1.3 gene is associated with low insulin sensitivity (Tschritter et al., 2005). Kv1.3 is highly expressed in the olfactory bulb along with TrkB and IR. We expected that this observed interaction between TrkB and Kv1.3 at the expression level may be of physiological importance as Kv1.3-null mice have increased expression of TrkB in the olfactory bulb (Fadool et al., 2004). The increase in Kv1.3 protein in the presence of TrkB suggests that in the native condition the levels of TrkB can alter the quantity of Kv1.3 expression and may suggest an explanation for the observed increase in TrkB protein in the olfactory bulb of Kv1.3 null mice. These experimental results also argue in favour of a relationship between expression levels of TrkB and the expression levels of Kv1.3. Kv1.3 and TrkB protein expression values also peak at different times (unpublished data) and this we suspect will also affect the kinetics of the observed increase in Kv1.3 expression with co-expression of TrkB. The finding that maximum expression levels of
Kv1.3 are reached when TrkB and Kv1.3 are in a 1:1 ratio is informative because we do not know the exact levels at which they co-exist in neurons relative to each other.

Even when increasingly larger cDNA quantities of Kv1.3 can be having toxic effects on cellular health and protein expression, TrkB can somehow rescue Kv1.3 and increase its expression level in the cell. This observation implies that TrkB can regulate the expression of this ion channel not only in healthy states but also when the health of the cell is challenged. In the native condition this mechanism the cell possesses can be used to regulate Kv1.3 expression and can explain the observed increase in TrkB protein in the olfactory bulb of Kv1.3 null-mice. These experimental results argue in favour of a relationship between expression levels of TrkB and the expression levels of Kv1.3. Kv1.3 and TrkB protein expression values also peak at different times (unpublished data) and this we suspect will affect the kinetics of the observed increase in Kv1.3 expression with co-expression of TrkB. Given that TrkB protein expression levels vary from postnatal day 1 to 60 in the olfactory bulbs of rats (Tucker and Fadool, 2002) it is possible that during development and maturation varying TrkB levels in olfactory bulb neurons can regulate the expression levels of Kv1.3 and intrinsic membrane properties therefore excitability of mitral and granule neurons. This mechanism can also become crucial in plasticity in the olfactory bulb and the integration of newly generated granule cells from the subventricular zone into olfactory bulb circuitry. We put forward the theory that this relationship is not isolated to the olfactory bulb because we have shown that TrkB co-immunoprecipitates with Kv1.3, Kv1.5 and IR both from the olfactory bulb and the cortex. Finding TrkB and the insulin receptor in a protein-protein interaction both in the olfactory bulb and the cortex was not anticipated. However there is overlap of the downstream signaling of TrkB and IR. TrkB and IR both use the Phosphatidylinositol 3 (PI3)- kinase pathway (Mizuno et al., 2003; Wick et al., 2003) and can perhaps be co-localized in domains of the cell or membrane because they share a common downstream signaling pathway. TrkB and IR kinase activity, when turned on by BDNF and insulin respectively, are known to target Kv1.3 tyrosine residues resulting in the suppression of Kv1.3 current (Fadool et al., 2000; Colley et al., 2004). Kv1.3 co-immunoprecipitates both with TrkB and IR, this indicates that they can be in close enough proximity to become associated. If TrkB and IR are in close proximity it will have implications for TrkB and IR signaling in the olfactory bulb but it also begs the question: is TrkB and IR signaling related in the regulation of metabolism, feeding and body weight? If this is the case, then the olfactory bulb; being such an
An unexpected finding was that the N-terminal GFP-tagged Kv1.3 channel does not exhibit increased expression in the presence of TrkB unlike the WT Kv1.3 and even the myc-tagged Kv1.3. The GFP-tagged channels were previously found to express functional channels on the surface with gating properties that were virtually indistinguishable from the WT Kv1.3 (Kupper, 1998). However presence of the N-terminus GFP-tag in Kv1.4 altered the gating kinetics of the channel. The T1 domain or first tetramerization domain is a highly conserved sequence in the cytosolic N-terminus of Kv channels (Deutsch, 2002). It is possible that the close proximity of the N-terminus GFP tag to the T1 domain of Kv1.3 may be impeding the stabilization of Kv1.3 by TrkB and thus the effect of increased expression in the presence of TrkB is lost. The cytoplasmic N-terminus region of Kv1.3 possesses a region rich in prolines (39-44: PLPPALP) and also two regions of tyrosine residues that are targets of phosphorylation of the TrkB receptor, the YYY111-113FFF and Y137F sites. It is possible the presence of the GFP may be distorting the usual folding in this region of the channel sufficiently to hamper regular interactions involving recognition of these sites as motifs for phosphorylation and events that may affect cytoskeletal and adaptor proteins interactions of the TrkB upregulation mechanism.

TrkB and Kv1.3 immunoreactivity overlap in the olfactory bulb mainly within the granule cell layer in what seems to be a distinct sub layer or lamina that lies more medial than on the periphery of the granule cell layer. TrkB and Kv1.3 also share localization in some fibers bordering the glomeruli or in the peri-gglomerular region. These fibers could be from centrifugal inputs into the olfactory bulb from higher brain regions or from the mitral and tufted cell axons exiting the olfactory bulb and making their way to the lateral olfactory tract (Shepherd et al., 2004). The co-localization of Kv1.3 and TrkB places the findings of this study into the context of the olfactory bulb and brain and imply that the relationship between TrkB and Kv1.3 has implications for modulating membrane properties and excitability and should be investigated further in vivo.
CHAPTER 3

Brain-derived neurotrophic factor (BDNF)-evoked modulation of Kv1.3 is perturbed by associated adaptor proteins, N-Shc and Grb10

Introduction

Adaptor proteins and kinases involved in the brain-derived neurotrophic factor (BDNF) pathway can form signaling complexes with membrane proteins such as Kv1.3 and become crucial interacting partners in shaping a neuron’s response to stimulation involving specific stimuli. Neuronal Shc (N-Shc) is an adaptor protein involved in downstream BDNF/TrkB signaling which is directly linked to neuronal survival (Atwal et al., 2000). Growth receptor binding protein 10 (Grb10) is also a downstream signaling molecule of the PI-3K/Akt (Jahn et al., 2002) a downstream pathway of the TrkB signal but the significance of Grb10 for TrkB signaling remains unknown. These same BDNF pathways are critical for survival and plasticity (Atwal et al., 2000, 2001). Our laboratory has shown that signaling molecules such as N-Shc and Grb10 can alter cellular tyrosine kinase modulation of Kv1.3 function through possible interactions with recognition motifs. Adaptor proteins such as N-Shc and Grb10 possess domains that can recognize and interact with Kv1.3 sequences containing phosphorylated tyrosines (Holmes et al., 1996a; Cook and Fadool, 2002). These sites become domains where these adaptor proteins can dock and recruit downstream signaling molecules activating the PI3K/Akt pathway (Whitehead et al., 2000) or the Raf1 and MEK1 kinase (Nantel et al., 1998; Atwal et al., 2000). Kv1.3 tyrosines are targets of TrkB kinase and can therefore be potential docking places for adaptor proteins that can themselves become activated by TrkB. The tyrosine kinase dimerizes upon activation, auto-phosphorylating tyrosines residues within its cytoplasmic domain thereby creating docking sites for adaptor proteins such as N-Shc. Our laboratory has also shown the presence of N-Shc and Grb10 in the olfactory bulb and that Grb10 can in fact significantly reduce v-Src-induced Kv1.3 phosphorylation, whereas tyrosine phosphorylation is
modestly increased in the presence of N-Shc (Cook and Fadool, 2002) but with an observed ease of v-Src-induced current suppression.

Kv1.3 is highly expressed in the olfactory bulb along with TrkB as well as N-Shc and Grb10 (Cook and Fadool, 2002). In addition N-Shc and Grb10 may be of physiological importance as Kv1.3 null-mice have increased expression of N-Shc, Grb10 and TrkB in the olfactory bulb (Fadool et al., 2004). I therefore hypothesized that if the absence of the Kv1.3 can lead to an increase in expression of N-Shc and Grb10 then the presence or over-expression of these adaptor proteins could be involved in BDNF modulation of Kv1.3 current. Hence, it seemed logical and important to investigate the possible functional roles of these adaptor proteins in relation to BDNF signaling and its modulation of Kv1.3.

BDNF signaling is important in the olfactory bulb as elsewhere in the brain and TrkB receptors are found within the olfactory bulb (Deckner et al., 1993) as well as Kv1.3 which has been shown to carry about 60-80% of outward current of olfactory bulb neurons (Fadool and Levitan, 1998). This makes the olfactory bulb an appropriate place to investigate possible significance of adaptor protein function in BDNF modulation of Kv1.3. The data in this chapter suggest that these adaptor molecules can relieve the suppression of Kv1.3 evoked by acute BDNF modulation of Kv1.3. Given the role of Kv1.3 in neurons and the time- and sensory-dependent effects of BDNF modulation of Kv1.3, this chapter characterizes how acute modulation of Kv1.3 can be altered by adaptor molecules highly expressed in the brain and involved in BDNF signaling downstream of the TrkB receptor.

This study investigates the effects of N-Shc and Grb10 on acute BDNF modulation of Kv1.3 in a heterologous expression system where I could isolate these key players of interest and measure their effects. Also investigated are the effects of PSD95, a structural protein with known roles in the recruitment of Kv1.4 channels and to a lesser extent Kv4.2 channels to lipid rafts (Wong and Schlichter, 2004). PSD-95 can also regulate Kv1 channel surface expression and clustering (Tiffany et al., 2000). PSD-95 has no known interaction in the downstream signaling pathway of BDNF but when over-expressed, like N-Shc and Grb10, relieved the acute BDNF current suppression of Kv1.3.
Materials and Methods

Solutions and Reagents. Human embryonic kidney cell (HEK 293) patch pipette solution contained (in mM): 30 KCl, 120 NaCl, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 2 CaCl₂ (pH 7.4). HEK 293 cell recording bath solution contained (in mM): 150 KCl, 10 HEPES, 1 Ethylene glycol-bis (β-amino-ethylether)-N, N', N'-tetraacetic acid (EGTA), and 0.5 MgCl₂ (pH 7.4). Cell lysis buffer with protease and phosphatase inhibitors (PPI solution) contained (in mM): 25 tris (hydroxymethyl) aminomethane (pH 7.5), 150 NaCl, 150 NaF, 0.5 Ethylenediaminetetraacetic acid (EDTA), 1 sodium orthovanadate (pH 8.0), 1 phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A and 1.0% Triton X-100. Nonidet-NP40 protease and phosphatase inhibitor (NP40 PPI) solution contained the same phosphatase inhibitors as above and in mM: 20 Tris base (pH 7.5), 150 NaCl, 1 % nonidet-NP40 and 10% glycerol. Wash buffer contained (in mM): 25 Tris base (pH 7.5), and 150 NaCl, 150 NaF, 0.5 EDTA and 0.1% Triton X-100. Homogenization buffer (HB) contained in (mM): 320 sucrose, 10 Tris Base, 50 KCl, and 1 EDTA (pH 7.8). Human recombinant brain-derived neurotrophic factor (rhBDNF) was purchased from Promega (Madison, WI). Tissue culture reagents were purchased from Gibco/BRL (Gaithersburg, MD). All salts were purchased from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Houston, TX).

cDNA Constructs and Antibodies. Kv1.3 channel was subcloned into the multiple cloning region of pcDNA3 (Invitrogen) using the unique restriction site HindIII. TrkB cDNA was a generous gift from Dr. P. Barker (Jahn et al., 2002) in a CMX vector. All channel and kinase coding regions were downstream from a cytomegalovirus (CMV) promoter. The 10-amino acid c-myc epitope (EQKLISEEDL) was inserted into the S1/S2 loop between residues 226-227 of Kv1.3 channel via two consecutive PCR reactions using Expand Long Template PCR System, Roche (Indianapolis, IN). Neuronal Shc (N-Shc) cDNA were generous gifts from Dr. T. Nakamura (Sumitomo Electric Industries, Yokohama, Japan) and were expressed in the vector pCMV1 (Nakamura et al., 1996). Grb10 cDNA was a gift from Dr. R. Roth (Stanford University) and was expressed in pBlueScript SK vector (Stratagene).
AU13, a rabbit polyclonal antiserum, was generated against a 46 amino acid sequence (478MVIEEGMNHASFPQTPFKTGNSTATCTTTNNPNDVCNIIKIFTDV523) representing the unique coding region of Kv1.3 on the C-terminus. Genmed Synthesis (San Francisco, CA) purified the peptide and Cocalico Biologicals (Reamstown, PA) the antiserum as previously characterized in Tucker and Fadool (Tucker and Fadool, 2002). This antibody was used for immunoprecipitation (1:1000), Western blot detection (1:5000) of Kv1.3 and immunohistochemistry (1:1000). Tyrosine phosphorylated proteins were visualized by the anti-phosphotyrosine antibody 4G10 from Upstate Biotechnology (Lake Placid, NY) and used at 1:1000 for Western blots. Tyrosine phosphorylated proteins were immunoprecipitated with 4G10 antibody (3 µg per 600-1200 µg of lysate). Monoclonal antiserum directed against amino acids 156-322 of human TrkB was purchased from Transduction Laboratories (San Diego, CA) and used at 1:1000 for Western blots. GRB10 (K-20) rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). SHC rabbit polyclonal antibody from BD Transduction Laboratories (San Diego, CA) was used at for immunohistochemistry (1:500) and Western Blotting (1:750). SHC antibody recognizes a peptide mapping from residues 359 to 473 of human SHC. Monoclonal Anti-β actin was purchased from Sigma (St. Louis, MO) and used at 1:750 for Western blots or rabbit polyclonal Anti-β actin from Sigma (A 2066) was used at 1:1000. Anti-c-myc mouse monoclonal (clone E910) to a peptide (EQKLISEEDL) from the human myc protein was purchased from Roche (Nutley, NJ) and used for Western blots. Donkey anti-rabbit FITC-conjugate (1:100) and goat anti-rabbit Texas Red-conjugate (1:200) were from Southern Biotechnology Association (Birmingham, AL).

HEK 293 Cell Culture and Transient Transfection. HEK 293 cells were maintained in Minimum Essential Medium (MEM), 2% penicillin/streptomycin, and 10% FBS (Gibco BRL). Cells were grown to 95% confluency dissociated with trypsin-EDTA (Sigma) and mechanical trituration, diluted in MEM to a concentration of around 600 cells/ml, and plated on Corning dishes (Catalog # 25000, Fisher Scientific). Before transfection, cells were grown to between 50-60% and 80-95% confluency for electrophysiology or biochemistry respectively. cDNA was introduced into HEK 293 cells with a Lipofectamine™ Transfection Reagent, Invitrogen (Carlsbad, CA) 3-5 days after passage as previously described (Fadool et al., 1997). Briefly, cells were transfected for 4-5 hrs using 2.0 µg of each cDNA construct per 60 mm dish.
or biochemistry or 4-5 hrs using 1.0 µg of each cDNA construct per 35 mm dish for physiology. Cells were then harvested for biochemistry or used for patch-clamp recording approximately 24-48 hrs post transfection.

**Electrophysiology.** Hoffman modulation contrast optics was used to visualize HEK 293 cells at 40X magnification (Axiovert 135, Carl Zeiss). Macroscopic currents in cell-attached membrane patches were detected using an Axopatch-200B amplifier (Axon Instruments). All electrophysiological data were recorded and analyzed using pClamp 8.0 software in combination with the analysis package of Origin, MicroCal Software (Northampton, MA) and Quattro Pro, Borland International (Scotts Valley, CA). Patch electrodes were fabricated from Jencons glass (Jencons Limited, Bedfordshire, UK), fire-polished to ~1 µm, and coated near the tip with beeswax to reduce the electrode capacitance. Pipette resistances were between 9 and 14 MΩ. The diameter of the patch pipette, and hence number of ion channels sampled, was held uniform by checking the bubble number of the pipette immediately after electrode fabrication and polishing (Mittman et al., 1987). In general, currents were recorded from cell-attached patches held at a holding potential (V_h) of −90 mV and stepped to +40 mV depolarizing potentials (V_c) with a pulse-duration of 1000 ms for Kv1.3. Pulses were delivered at 45 s intervals in order to prevent cumulative inactivation of Kv1.3 channels. Tail currents for voltage at half-activation was obtained by stepping from −90 mV to −5 mV in 5 mV increments for durations of 50 ms every 10 s. The time points measured for tail currents were pre: 8 minutes, during: 13 minutes and post: 22 minutes. The voltage step depolarization lasted about 5 minutes and was followed by a 30 – 45 s interpulse interval after which tail currents were measured for 3 minutes and the cycle repeated. These two pairs of protocols were run for a total of 3 times lasting about 23 – 25 minutes in total duration to obtain pre, during and post BDNF stimulation parameters for Kv1.3.

Peak outward current amplitude, time constants of channel inactivation and deactivation were determined by parameter fitting for BDNF pre-stimulation 5 min (time 0), 10 min mid-stimulation and 20 min post-stimulation. The inactivation time constant was determined by fitting the current trace activated by a −90 mV to +40 mV depolarizing step with a two-term exponential standard function (using Chebyshev’s technique in Clampfit 8.0 software) according to the following equation:

\[ I(t) = A_1 \exp\left(-t/\tau_1\right) + A_2 \exp\left(-t/\tau_2\right) + C \]
where $A_1$ and $A_2$ are the amplitudes, $\tau_1$ and $\tau_2$ are the time constants and $C$ is the offset constant. The inactivation time constant was calculated as the weighted average of the two time constants. Deactivation time constant was determined by the fit to one-term using the above exponential standard function to the tail current. Tail current amplitudes were plotted in a current-voltage relationship and fit to a Boltzmann sigmoidal curve to calculate the slope of a voltage dependence ($\kappa$) and voltage at half-activation ($V_{1/2}$) for Kv1.3. Differences in these biophysical measurements were analyzed between pre (time 0 min), during (time 10 min) and post-growth factor stimulation (time 20 min) groups using repeated measure ANOVA within groups and one-way ANOVA to compare between selected groups with statistical significance at the 95% confidence level followed by the Tukey-Kramer post tests. BDNF stimulation was accomplished by tip-filling (~0.01 mm) the patch-pipette with control solution and then back filling (~35 mm) with BDNF (1 ng/µl). Insulin (10 ng/ml) stimulation was done the same as for BDNF. The dilution of the BDNF/insulin using this procedure would be insignificant due to the ratio of the volume (back-fill/tip-fill) difference that estimated to be at least 1 million.

**Immunocytochemistry.** Twenty-four to 48 hours after transfection, cells were fixed with 1% paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes at room temperature. This was followed by three 10-minute washes with PBS. Afterwards, aspecific protein binding was blocked with 30 minutes incubation in 2% bovine serum albumin (BSA) in PBS. Surface c-myc-tagged channels were labeled at room temperature for 90 minutes with mouse c-myc antibody at 1:800 dilution in 2% BSA in PBS. This was followed by three 10-minute washes in PBS and the permeabilization in 2% BSA in 0.1% Triton X-100 and PBS (PBST) for 45 minutes. Subsequent incubation with fluorescent secondary antibody, Texas Red-conjugated goat anti-mouse (1:100), was performed in the dark for 90 minutes. This was followed by a wash with PBST, five minutes incubation with diamidino-phenyindole (DAPI) in PBS (1:5000) and a final wash in PBS. Coverslips were mounted onto slides with 70/30% glycerol/PBS, containing p-phenylene-diamine.

**Immunohistochemistry.** C57 mice (n =3) at postnatal day 20-30 were anaesthetized with an overdose of sodium pentobarbital and perfused transcardially with ice-cold phosphate buffered saline (PBS; pH 7.4) followed by ice-cold 4% paraformaldehyde. Olfactory bulbs were
dissected out and post fixed in the same fixative for 4 hours at 4°C. The tissue was cryoprotected in 30% sucrose overnight, sectioned on a cryostat at 10 µm thickness in the coronal plane and mounted onto 2 % gelatine coated slides. Sections were stored at –20°C until used. Sections were air-dried for 30 minutes and rinsed 3 times with 1x PBS (ph 7.4) every 5 minutes. Sections were then incubated for 30 minutes in blocking solution made with 10 % Goat Serum in PBS containing 0.3% Triton-X-100. The blocking solution was then drained and any excess blotted off the slide. Primary antibody was diluted in blocking solution and applied to the sections for 2 hours at room temperature or overnight at 4°C. The sections were then rinsed 3 times in PBS every 5 minutes and the secondary antibody applied in blocking solution as above for 2 hours at room temperature or overnight at 4°C. The sections were handled in the dark from hereon. The double labeling experiments were performed by repeating one more round of labeling using another primary against the second protein (Shc or Grb10) to show colocalization. The sections were then given a final rinse in PBS for 5 minutes followed by a 5 minute rinse in distilled water and mounted using Vectasheild. The edges of the cover slip were sealed and slides were stored at 4°C until imaging. Control slides were treated exactly the same except the primary antibodies were omitted. Sequential labeling was done using AU13 as the first primary antibody; to be labeled with FITC-conjugate, then followed by anti-SHC or anti-GRB10. Anti-SHC and anti-GRB10 were then labeled with Texas Red-conjugate for the appropriate experiments.

Confocal Microscopy and Image Analysis. Microscopic analysis of fluorescently labeled cells was performed on a Zeiss Axioplan 2 Microscope attached to a Zeiss LSM510 confocal system (Carl Zeiss, Germany). Samples were excited at 488 nm (for GFP) and 543 nm (for Texas Red) through a 63x oil-immersion objective (NA=1.40) and fluorescence between 500-545 and 565-615 nm was detected from <0.8 µm thick optical sections. Images were taken from the cells at their maximal cross section. Cell outlines were identified using the Texas Red staining.

Immunoprecipitation and Electrophoretic Separation. Olfactory bulbs were quickly harvested from C57-BJ6 mice at P20 after decapitation. Strict adherence was kept to American Veterinary Medical Association- and National Institutes of Health-approved methods. Olfactory
bulbs were immediately homogenized in homogenization buffer (HB) with a Kontes tissue grinder (size 20; 50 strokes) on ice. The mixture was centrifuged twice at ~2400 g (3800 RPM) for 30 minutes at 4°C in an Eppendorf model 5416 to remove cellular debris. The supernatant was then centrifuged in a Beckman Ultracentrifuge (model L7, Beckman, Westbury, NY) at 100,000 g (40,000 RPM) for 2 hours at 4°C. The resulting pellet was resuspended in HB solution and gently resuspended using a Kontes hand held homogenizer for 2-5 seconds. Protein concentration was determined in triplicates by Bradford assay and samples were stored at -80°C until use. For immunoprecipitation, freshly harvested OB or cortex was homogenized using a Kontes tissue grinder (size 20; 50 strokes) in ice-cold HB containing 0.5% Nonidet P-40 detergent in PPI solution (see “Solutions and Reagents”). Lysis was continued on a Roto-Torque slow speed rotary (model 7637; Cole Palmer, Vernon Hills, IL) for 10 minutes at 4°C. The lysates were clarified by centrifugation at 14,000 X g for 10 minutes at 4°C and precleared for 1 hour with 3 mg/ml protein A-sepharose (Amersham-Pharmacia, Newark, NJ), which was followed by another centrifugation step to remove the protein A-sepharose. Lysates were obtained from transfected HEK 293 cells as previously described (Colley et al., 2004). Clarified lysates were incubated overnight at 4°C with 3 µg of respective antibody per 600-1200 µg of HEK 293 cells or 2000-3000 µg native tissue lysate. Immunoprecipitates were then collected after a 2 hour incubation with protein A-sepharose, centrifuged as above and washed 4 times with ice-cold NP40 PPI solution for native tissue or with ice-cold wash buffer for HEK 293 cells. Lysates and washed immunoprecipitates were diluted 1:1 in sodium dodecyl sulfate (SDS) gel loading buffer (Sambrook et al., 1989) containing 1 mM Na3VO4 and stored at –20°C for subsequent analysis. Membrane proteins or immunoprecipitated proteins from native tissue or transfected HEK 293 cells were separated on 10% acrylamide gels by SDS-PAGE and electro-transferred to nitrocellulose blots. Blots were blocked with 5% nonfat milk and incubated overnight at 4°C in primary antisera against Kv1.3 or Kv1.5, TrkB, IRβ or 4G10 antibody. They were then incubated with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit secondary (1:6000) (Amersham-Pharmacia) or HRP-conjugated goat anti-mouse secondary (1:6000) (Sigma) for 60 minutes at room temperature. Enhanced chemiluminescence (ECL; Amersham-Pharmacia) exposure on Fugi Rx film was used to visualize labeled proteins. The film autoradiographs were analyzed by quantitative densitometry using a Hewlett-Packard PhotoSmart Scanner (model 106-816, Hewlett Packard, San Diego, CA) in conjunction with
Quantiscan software (Biosoft, Cambridge, England). The raw densitometry values for the different groups were obtained from the same autoradiograph and therefore same exposure time for each batch of experiments and then analyzed using a Student’s t-test.

**Immunoprecipitation of surface myc-tagged Kv1.3.** HEK293 cells transfected with mycKv1.3 -/+ TrkB, were rinsed once with 1x PBS at room temperature and incubated for 30 minutes with 0.4 µg/µl mouse monoclonal c-myc antibody (1:400) in HEK 293 patch pipette solution (see Reagents and Solutions). The cells were rinsed in PBS and then BDNF (100 µg/ml) in HEK 293 patch pipette (30 mM K⁺) solution was applied for 10 minutes at room temperature then rinsed with PBS. All conditions were performed to best replicate conditions of the cell-attached recording conditions. The cells were then lysed and myc- labeled surface Kv1.3 proteins were immunoprecipitated as stated in immunoprecipitation methods above.

**Results**

**Kv1.3, TrkB, N-Shc, Grb10 and PSD95 contain potential interaction sites.** Figure 3.1 shows the positions of tyrosine residues on Kv1.3 that can become phosphorylated: 111-113, 137, 449 and 479. TrkB kinase activity leads to phosphorylation of tyrosine residues 111-113, 137 and 449 in Kv1.3 (Colley et al., 2004). These phosphorylated tyrosines can then form protein-protein interactions with the SH2 domains of N-Shc and Grb10 depicted in Figure 3.1. SH2 domains recognize short sequences of tyrosine phosphorylated residues in target proteins such as Kv1.3 (Pawson and Scott, 1997). Other potential sites of interaction include proline rich regions in the N (39-44) and C (493-496) termini of Kv1.3 that SH3 domains recognize. PSD95 can interact with Kv1.3 via proline rich regions or via recognition of the PDZ recognition motif KIFTDV (520-525) in the N terminus of Kv1.3.
Figure 3.1 Representation of interacting domains of Kv1.3, TrkB, N-Shc, Grb10 and PDS95.

Kv1.3 distribution pattern in the olfactory bulb of mice overlaps with Shc and Grb10. In Figure 3.2 (I), while Kv1.3 immunoreactivity was seen throughout the layers of the olfactory bulb with the exception of the outer nerve layer, the highest Shc staining was seen mainly in fibers surrounding the glomeruli and passing through the internal plexiform layer (IPL). It is in these regions of the glomerular layer (GL) and the IPL that there is strong overlap with Kv1.3 distribution pattern. There is a weaker and more dispersed overlap of Shc in some fibers of the external plexiform layer (EPL) with Kv1.3.

In Figure 3.2 (II), Grb10 had the strongest immunosignal almost throughout the entire granule cell layer in the olfactory bulb and overlaps strongly in fibers within this layer with Kv1.3 signal as well as in some fibers passing through the IPL. Grb10 signal appears to be more punctate than that of the Shc signal but this may be an artifact of the exact angle the cross-sectional slice takes through the structure of the olfactory bulb. Observe that the negative sections show no signal in the absence of primary antibody for Kv1.3 and Shc in Figure 3.2 I and no signal for Kv1.3 and Grb10 in Figure 3.2 II.
Adaptor proteins N-Shc, Grb10, and PSD95, perturb acute BDNF-evoked current suppression of Kv1.3 and shift Kv1.3 voltage at half-activation. Acute BDNF application suppresses Kv1.3 current (Colley et al., 2004) and this is shown in Figure 3.3 B a representative example of the 5th and 20th minute traces as shown in Table 3.1a. The mean suppression was 20% when calculated as the average ratio of the peak current amplitude of the 20th minute/5th minute trace. Repeated measures ANOVA was performed for each condition to test if there were any significant changes in peak current amplitude, inactivation and deactivation kinetics within cells pre (a), during (b) and post (c) treatment with BDNF by using the measurements taken at the 5th, 10th and 20th minute time intervals. No other significant differences were found besides the difference from the 5th to the 20th minute of BDNF-induced Kv1.3 current suppression in the Kv1.3 + TrkB condition. For the purpose of understanding any significant changes from the 5th to the 20th minute a simple paired t test was used within the conditions and the inactivation time constant of Kv1.3 in the Kv1.3 + TrkB + Grb10 from the 5th to the 20th minute was found to have changed significantly at $\alpha \leq 0.05$, at the two-tail level. BDNF current suppression, however, did not occur in the presence of over-expressed N-Shc, Grb10 or PSD95 and a One-way Analysis of Variance (ANOVA) between KT and KTS, KTG and KTP found the variance amongst the means was not greater than that expected by chance alone. Also, none of the BDNF treatment conditions had any significant differences, in the biophysical properties measured and shown in Table 3.1a, from that of their matched vehicle control cells.

Kv1.3 + TrkB control parameters measured and displayed in Table 3.1b were not different from that of BDNF treated cells when an ANOVA was run to compare them neither was there any significant differences found with the means of BDNF-vehicle control cells Kv1.3 + TrkB + N-Shc (KTS) or + Grb10 (KTG) or + PSD95 (KTP). However, ANOVA comparison of the means for BDNF post-treated KT, KTS, KTG and KTP revealed by Tukey-Kramer post-test that the $V_{1/2}$ mean of KTS was significantly different from that of at $\alpha \leq 0.01$ (**) and KTP. As shown in Figure 3.4 F, KTG and KTP shift $V_{1/2}$ significantly to the left compared to KTS. Comparing pre-BDNF, KTS was significantly different from KTP at $\alpha \leq 0.05$ (*). The repeated measure ANOVA tests done to compare within cell pre, during and post measures found as shown in the table that mean $V_{1/2}$ measures for KTG, pre compared to post was significantly at $\alpha \leq 0.01$ and KTP, pre to post was significantly different $\alpha \leq 0.05$. The only
significant difference within cell for the voltage at half-activation slope (κ) was for KTP, an increase from the pre to the post at $\alpha \leq 0.05$.

Figure 3.2 Co-localization of N-Shc and Grb10 with Kv1.3 in the olfactory bulb of mice. C57 mice were anaesthetized at day postnatal day 20-30 and perfused. Olfactory bulbs were dissected, post-fixed, cryoprotected, sectioned on a cryostat at 10 μm in the coronal plane and mounted on 2% gelatin coated slides. Sections were incubated with Kv1.3 antibody then with secondary antibody for the green channel followed by sequential labeling with antibody against Shc or Grb10 then antibody for the red channel. The control sections were treated exactly the same except the primary antibodies were excluded. I, Kv1.3 labeling in the olfactory bulb layers seen in the green channel (A, D, G) and N-Shc seen in the red channel (B, E, H). The merger of the two shows overlapping regions as yellow (C, F, I). II, Grb10 labeling in the olfactory bulb layers seen in the red channel (A, D, G) and Kv1.3 seen in the green channel (B, E, H). The merger of the two shows overlapping regions as yellow (C, F, I). Starting from the exterior circumference of the olfactory bulb where the outer nerve layer (ONL) lies, immunofluorescence is seen to different extents in the sections in I and II, in fibers of the glomerular layer (GL), external plexiform layer (EPL), mitral cell layer (MCL), internal plexiform layer (IPL) and the
granule cell layer (GCL). In I, Kv1.3 and Shc overlap in the peripheral structures surrounding the glomeruli and in the EPL, MCL, IPL and GCL. In II, Kv1.3 and Grb10 overlap mainly in the IPL, GCL and slightly in some fibers surrounding the glomeruli.

Figure 3.3 BDNF-induced activation of TrkB receptor causes current suppression of Kv1.3 that is relieved by adaptor proteins N-Shc and Grb 10 and by post-synaptic density protein PSD95. HEK 293 cells were transfected with Kv1.3 and TrkB alone (A, B) or with either N-Shc (C), Grb10 (D) or PSD95 (E). The patches were held at \( V_h \) −90 mV and stepped to a single depolarizing pulse of +40 mV \( V_c \) for 1000 ms every 45 s. Patch pipettes were tip filled with normal patch solution and then back filled with 1 ng/μl BDNF in order to present the ligand to the external surface of the membrane. The 5th minute trace is used as the first or pre-stimulation measurement and follows stabilization of the patch (1-3 minutes). The neurotrophin-treated trace is from the same patch recorded 20 minutes later (20th minute trace) or the post-stimulation trace. A, representative recording from a BDNF-vehicle control cell condition for which the pipette was back-filled with 1 ng/μl of OptiMEM. B-E show representative recordings taken
from cell membrane patches exposed to BDNF. F, histogram representing the 20th minute peak current amplitude normalized to the 5th minute peak current amplitude for each of the conditions shown in A-E. * = BDNF-treated (20th minute) is significantly different from the pre-treated (5th minute) by a paired t-test, $\alpha \leq 0.05$.

Table 3.1a Effects of N-Shc, Grb10 and PSD95 on peak current, inactivation and deactivation properties of acute-BDNF modulation of Kv1.3 current.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Peak Current (pA)</th>
<th>$t_{\text{inact}}$ (ms)</th>
<th>$t_{\text{deact}}$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kv1.3 + TrkB Control</td>
<td>Pre (a) 551 ± 198 (4)</td>
<td>788 ± 186</td>
<td>26.8 ± 8.0</td>
</tr>
<tr>
<td></td>
<td>During (b) 556 ± 179</td>
<td>753 ± 104</td>
<td>19.9 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>Post (c) 557 ± 183</td>
<td>742 ± 130</td>
<td>24.9 ± 4.4</td>
</tr>
<tr>
<td>Kv1.3 + TrkB</td>
<td>Pre 555 ± 173 (7)</td>
<td>900 ± 243</td>
<td>26.8 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>During 532 ± 154</td>
<td>687 ± 118</td>
<td>24.7 ± 4.8</td>
</tr>
<tr>
<td></td>
<td>Post 472 ± 173* (a,c)</td>
<td>762 ± 151</td>
<td>28.8 ± 6.7</td>
</tr>
<tr>
<td>Kv1.3 + TrkB + N-Shc</td>
<td>Pre 343 ± 71 (13)</td>
<td>618 ± 67</td>
<td>27.4 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>During 370 ± 81</td>
<td>659 ± 79</td>
<td>26.1 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>Post 381 ± 92</td>
<td>549 ± 68</td>
<td>26.4 ± 2.0</td>
</tr>
<tr>
<td>Kv1.3 + TrkB + Grb10</td>
<td>Pre 488 ± 114 (7)</td>
<td>862 ± 110</td>
<td>19.3 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>During 537 ± 124</td>
<td>788 ± 63</td>
<td>24.9 ± 5.2</td>
</tr>
<tr>
<td></td>
<td>Post 545 ± 133</td>
<td>553 ± 46* (a,c)</td>
<td>25.4 ± 5.5</td>
</tr>
<tr>
<td>Kv1.3 + TrkB + PSD95</td>
<td>Pre 231 ± 46 (6)</td>
<td>552 ± 115</td>
<td>30.1 ± 16.1</td>
</tr>
<tr>
<td></td>
<td>During 224 ± 44</td>
<td>530 ± 83</td>
<td>34.0 ± 9.0</td>
</tr>
<tr>
<td></td>
<td>Post 225 ± 53</td>
<td>779 ± 211</td>
<td>26.3 ± 7.4</td>
</tr>
</tbody>
</table>

HEK 293 cells were transfected as treatment conditions shown in the table. Kv1.3 currents were recorded in cell-attached membrane patches held at a holding potential ($V_h$) of $–90$ mV and stepped to $+40$ mV depolarizing potentials ($V_c$) with a pulse-duration of 1000 ms. Pulses were delivered in 45 s intervals to prevent cumulative inactivation and a total of 3 sets of this protocol was done with 8 steps per set interspersed by stepped depolarizations as in Table 3.1b. Paired t-test was used to compare pre to post measures, Repeated measures ANOVA and Tukey-Kramer post test were used to compare pre (a), during (b) and post (c) stimulation parameters within treatment conditions with significance level at $\alpha \leq 0.05$. Significantly different pre, during and post measurements are denoted in parenthesis within the table. Pre: 5th minute, during: 10th minute and post: 20th minute.
Figure 3.4 Voltage at half-activation ($V_{1/2}$) is left-shifted in the presence of Grb10 and PSD95 and restored in the presence of N-Shc. HEK 293 cells were transfected with Kv1.3 and TrkB alone (A, B) or with either N-Shc (C), Grb10 (D) or PSD-95 (E) as in Figure 3.3. Cell-attached membrane patches were voltage-clamped with $V_h$ at $-90$ mV and $V_c$ stepped from $-90$ to $-5$ mV in $5$ mV increments with a pulse duration of $50$ ms and interpulse interval at $10$ s. F, peak tail-current amplitudes were normalized to the current measured at $-5$ mV and plotted against voltage. Points were fit to a Boltzmann function. Arrow, note left-shifted voltage at half-activation produced in the presence of BDNF + Grb10 and BDNF + PSD95 which is restored in the presence of N-Shc.

Acute BDNF stimulation of TrkB lacking the shc- site (TrkBshc-) results in current suppression that is significant when comparing the $5^{th}$ minute to the $20^{th}$ minute like our analysis done in
Table 3.1b Effect of N-Shc, Grb10 and PSD95 on voltage at half-activation measurements of acute-BDNF modulated Kv1.3 current.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$V_{1/2}$</th>
<th>$\kappa$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kv1.3 + TrkB Control</td>
<td>Pre (a)</td>
<td>-30.4 ± 5.0 (4)</td>
</tr>
<tr>
<td></td>
<td>During (b)</td>
<td>-27.6 ± 6.0</td>
</tr>
<tr>
<td></td>
<td>Post (c)</td>
<td>-28.6 ± 5.8</td>
</tr>
<tr>
<td>Kv1.3 + TrkB</td>
<td>Pre</td>
<td>-36.2 ± 4.6 (7)</td>
</tr>
<tr>
<td></td>
<td>During</td>
<td>-26.4 ± 6.1</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>-40.3 ± 6.7</td>
</tr>
<tr>
<td>Kv1.3 + TrkB + N-Shc</td>
<td>Pre</td>
<td>-30.9 ± 1.9 (13)</td>
</tr>
<tr>
<td></td>
<td>During</td>
<td>-33.6 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>-33.5 ± 2.9</td>
</tr>
<tr>
<td>Kv1.3 + TrkB + Grb10</td>
<td>Pre</td>
<td>-42.6 ± 3.7 (7)</td>
</tr>
<tr>
<td></td>
<td>During</td>
<td>-48.8 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>-54.1 ± 2.8** (a:c)</td>
</tr>
<tr>
<td>Kv1.3 + TrkB + PSD95</td>
<td>Pre</td>
<td>-47.1 ± 2.7 (4)</td>
</tr>
<tr>
<td></td>
<td>During</td>
<td>-49.2 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>-52.8 ± 1.4* (a:c)</td>
</tr>
</tbody>
</table>

HEK 293 cells were transfected as for treatment conditions shown in the table. Kv1.3 currents were recorded in cell-attached membrane patches held at a holding potential ($V_h$) of −90 mV and stepped to -5 mV by 5 mV step depolarizations ($V_c$) with a pulse-duration of 50 ms and 10 ms interpulse intervals between protocol in Table 3.1a for a total of 3 times. Repeated measures ANOVA and Tukey-Kramer post test was used to compare pre (a), during (b) and post (c) stimulation parameters within treatment conditions with significance level at $\alpha = 0.05$. Significantly different pre, during and post measurements are denoted in parenthesis within the table.

Colley et al, 2004 using paired $t$ test but less pronounced than in the presence of TrkB see Figure 3.5 B and C. The on average current suppression ratio obtained with TrkBshc- is less than when TrkB is activated, 10 ± 4 % (10) compared to 18 ± 5 % (7). However, when doing repeated measures ANOVA with the 5th, 10th and 20th minute for the TrkBshc- condition we lose the significance of the effect perhaps due to variability added by the middle time point. Kv1.3 and TrkB retains the significance of current suppression even when comparison is done using all three time points and so does Kv1.3 + TrkBshc- + Grb10 (KTshc-G). KTshc-G shows an even more pronounced degree of current suppression 29 ± 3 % than KT. For the purpose of simplicity
of interpretation of the analysis we will use the paired t test as the choice of comparison because we do not expect the absence of the shc site to affect the activation of the TrkB kinase and subsequent effect on Kv1.3 which we found to involve phosphorylation of tyrosine residues (Colley et al., 2004). It does not exclude the possibility that the tertiary structure of TrkBshc- may be different enough from TrkB to hinder the usual interactions that follow on activation on the TrkB kinase that may require a specific conformation of TrkB.

The presence of Grb10 shifted the voltage at half-activation of Kv1.3 to less depolarized potentials and significantly so for BDNF treated Kv1.3 + TrkBshc- (KTshc-) to Kv1.3 + TrkBshc- + Grb10 (KTshc-G) at all time points pre, during and post was different when tested by one-way ANOVA and Tukey-Kramer’s post test at $\alpha = 0.001$ (***)). Comparison of within cell time points by repeated measure ANOVA for the various conditions suggests that there are no significant changes in $V_{1/2}$ and $\kappa$ caused by the treatment with BDNF. I was also interested in whether having TrkB vs TrkBshc- present with Kv1.3 would make a difference for both the vehicle control and BDNF and whether adding Grb10 to Kv1.3 would have an effect for both vehicle control and BDNF stimulated conditions. I compared these groups using one-way ANOVA for the values obtained for a particular measure eg: $V_{1/2}$ but found that they did not have significant effects.

**Adaptor proteins Grb10 and N-Shc differentially regulate Kv1.3 expression and phosphorylation.** Given the effects of N-Shc and Grb10 on BDNF induced current suppression of Kv1.3 we tested whether these adaptor proteins may be disrupting phosphorylation of the channel by TrkB. We repeated the transfection conditions to match the electrophysiology and tested the level of phosphorylation of Kv1.3 in the presence and absence of the adaptor proteins in BDNF stimulated and non-stimulated experiments to match the pre and post treatment conditions. Kv1.3 was then immunoprecipitated from equal weights of lysate from each condition and analyzed by Western blotting using anti-phosphotyrosine antibody (anti-pY) see Figure 3.7 A. Statistical analysis of 3 such experiments, as shown in Figure 3.7 A, found that the differences in phosphorylation of Kv1.3 levels in the cell in the + BDNF versus – BDNF conditions, in both KT and KTS, were still significantly different using $t$-tests run on the log of the + to - BDNF densitometry values. The expression level of Kv1.3 was very low when co-expressed with Grb10 and this is analyzed in Figure 3.8 A and B, so measuring changes in
Figure 3.5  BDNF-induced activation of TrkBshc- receptor exhibits significant current suppression of Kv1.3 that is greater in the presence of Grb10. HEK 293 cells were transfected with Kv1.3 and TrkB (A, B), Kv1.3 and TrkBshc- site alone (C) or with Grb10 (D) and Kv1.3 with Grb10 (E) or without Grb10 (F). Cell-attached membrane patches were held at \( (V_h) \) –90 mV and stepped to a single depolarizing pulse of +40 mV \( (V_c) \) for 1000 ms every 45 s. Patch pipettes were tip filled with normal patch solution and then back filled with 1 ng/\( \mu l \) BDNF in order to present the ligand to the external surface of the membrane. The 5\textsuperscript{th} minute trace is used as the first or pre-stimulation measurement and follows stabilization of the patch (1-3 minutes). The neurotrophin-treated trace is from the same patch recorded 20 minutes later (20\textsuperscript{th} minute trace) or the post-stimulation trace. A, shows a representative recording from a BDNF-vehicle control cell condition for which the pipette was back-filled with 1 ng/\( \mu l \) of OptiMEM. B-F show representative recordings taken from cell membrane patches exposed to BDNF. G, shows a histogram representing the 20\textsuperscript{th} minute peak current amplitude normalized to the 5\textsuperscript{th} minute peak current amplitude for each of the conditions shown in A-E. * = BDNF-treated (20\textsuperscript{th} minute) is significantly different from the pre-treated (5\textsuperscript{th} minute) by a paired t-test, \( \alpha \leq 0.05 \).
Table 3.2a Effect of TrkBshc- and Grb10 on peak current, inactivation and deactivation properties of acute-BDNF modulation of Kv1.3 current.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Peak Current (pA)</th>
<th>$\tau_{\text{inact}}$ (ms)</th>
<th>$\tau_{\text{deact}}$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kv1.3 + TrkB</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>431 ± 119 (5)</td>
<td>764 ± 170</td>
<td>37.2 ± 10.9</td>
</tr>
<tr>
<td></td>
<td>495 ± 112</td>
<td>1321 ± 332</td>
<td>26.9 ± 4.6</td>
</tr>
<tr>
<td></td>
<td>445 ± 132</td>
<td>1118 ± 581</td>
<td>32.3 ± 3.3</td>
</tr>
<tr>
<td><strong>Kv1.3 + TrkB</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre (a)</td>
<td>356 ± 71 (5)</td>
<td>697 ± 130</td>
<td>22.2 ± 2.1</td>
</tr>
<tr>
<td>During (b)</td>
<td>328 ± 72</td>
<td>814 ± 47</td>
<td>17.7 ± 2.5</td>
</tr>
<tr>
<td>Post</td>
<td>298 ± 64**(a,c)</td>
<td>780 ± 59</td>
<td>14.2 ± 2.9</td>
</tr>
<tr>
<td><strong>Kv1.3 + TrkBshc-</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>372 ± 144 (3)</td>
<td>692 ± 45</td>
<td>21.8 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>375 ± 139</td>
<td>660 ± 265</td>
<td>23.2 ± 10.1</td>
</tr>
<tr>
<td></td>
<td>386 ± 140</td>
<td>843 ± 114</td>
<td>16.5 ± 3.7</td>
</tr>
<tr>
<td><strong>Kv1.3 + TrkBshc-</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre (a)</td>
<td>533 ± 109 (10)</td>
<td>1070 ± 163</td>
<td>21.2 ± 4.3</td>
</tr>
<tr>
<td>During (b)</td>
<td>524 ± 105</td>
<td>873 ± 152</td>
<td>20.0 ± 3.4</td>
</tr>
<tr>
<td>Post</td>
<td>503 ± 115</td>
<td>1040 ± 196</td>
<td>25.3 ± 3.8</td>
</tr>
<tr>
<td><strong>Kv1.3 + TrkBshc-</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Grb10</td>
<td>533 ± 122 (4)</td>
<td>894 ± 173</td>
<td>19.0 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>650 ± 158</td>
<td>1098 ± 292</td>
<td>19.4 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>379 ± 84**(b,c)</td>
<td>1660 ± 229</td>
<td>32.6 ± 7.8</td>
</tr>
<tr>
<td><strong>Kv1.3 + Grb10</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre (a)</td>
<td>506 ± 271 (3)</td>
<td>774 ± 110</td>
<td>21.3 ± 9.5</td>
</tr>
<tr>
<td>During (b)</td>
<td>520 ± 267</td>
<td>774 ± 184</td>
<td>13.5 ± 1.3</td>
</tr>
<tr>
<td>Post</td>
<td>498 ± 246</td>
<td>610 ± 127</td>
<td>24.2 ± 9.7</td>
</tr>
<tr>
<td><strong>Kv1.3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre (a)</td>
<td>287 ± 49 (4)</td>
<td>1024 ± 252</td>
<td>21.5 ± 5.4</td>
</tr>
<tr>
<td>During (b)</td>
<td>309 ± 61</td>
<td>794 ± 272</td>
<td>22.8 ± 6.7</td>
</tr>
<tr>
<td>Post</td>
<td>303 ± 68</td>
<td>961 ± 363</td>
<td>38.3 ± 13.2</td>
</tr>
</tbody>
</table>

HEK 293 cells were transfected as treatment conditions shown in the table. Kv1.3 currents were recorded in cell-attached membrane patches held at a holding potential ($V_h$) of –90 mV and stepped to +40 mV depolarizing potentials ($V_C$) with a pulse-duration of 1000 ms. Pulses were delivered in 45 s intervals to prevent cumulative inactivation and a total of 3 sets of this protocol was done with 8 steps per set interspersed by stepped depolarizations as in Table 3.2b. Paired t-test was used to compare pre to post measures, Repeated measures ANOVA and Tukey-Kramer post test were used to compare pre (a), during (b) and post (c) stimulation parameters within treatment conditions with significance level at $\alpha \leq$ 0.05. Significantly different pre, during and post measurements are denoted in parenthesis within the table.
Figure 3.6 Voltage at half-activation ($V_{1/2}$) is left-shifted for Kv1.3 and TrkBshc- in the presence of Grb10. HEK 293 cells were transfected with Kv1.3 and TrkB alone (A, B) or with either nShc (C), Grb10 (D) or PSD-95 (E) as in Figure 3.3. Cell-attached membrane patches were voltage-clamped with $V_h$ at –90 mV and $V_c$ stepped from –90 to –5 mV in 5 mV increments with a pulse duration of 50 ms and interpulse interval at 10 s. F, peak tail-current amplitudes were normalized to the current measured at –5 mV and plotted against voltage. Points were fit to a Boltzmann function. Arrow, note left-shifted voltage at half-activation produced in transfection conditions in D, E and F.

phosphorylation of this reduced level of Kv1.3 was technically difficult. The phosphorylation signal for Kv1.3 in the Kv1.3 alone condition was expected to be very low and we did not expect to see phosphorylation of Kv1.3 in the condition with TrkB lacking its kinase (D) which confirmed to us that TrkB kinase activity does cause an increase in Kv1.3 phosphorylation.
Table 3.2b Effect of TrkBshc- and Grb10 on voltage at half-activation properties of acute-BDNF modulation Kv1.3 current.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pre (a)</th>
<th>During (b)</th>
<th>Post (c)</th>
<th>V_{1/2}</th>
<th>κ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kv1.3 + TrkB</td>
<td></td>
<td></td>
<td></td>
<td>-18.3 ± 1.4</td>
<td>-4.5 ± 0.3</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td>-17.6 ± 2.0</td>
<td>-4.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-19.4 ± 3.3</td>
<td>-5.9 ± 1.5</td>
</tr>
<tr>
<td>Kv1.3 + TrkB</td>
<td></td>
<td></td>
<td></td>
<td>-22.2 ± 2.1</td>
<td>-4.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-17.7 ± 2.5</td>
<td>-4.0 ± 0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-14.2 ± 2.9</td>
<td>-4.7 ± 0.3</td>
</tr>
<tr>
<td>Kv1.3 + TrkBshc-</td>
<td></td>
<td></td>
<td></td>
<td>-28.4 ± 9.5</td>
<td>-5.2 ± 0.8</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td>-28.4 ± 9.5</td>
<td>-4.3 ± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-31.1 ± 10.1</td>
<td>-1.8 ± 3.4</td>
</tr>
<tr>
<td>Kv1.3 + TrkBshc-</td>
<td></td>
<td></td>
<td></td>
<td>-19.6 ± 1.1</td>
<td>-4.2 ± 1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-19.7 ± 1.3</td>
<td>-3.8 ± 1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-19.9 ± 1.4</td>
<td>-4.0 ± 0.9</td>
</tr>
<tr>
<td>Kv1.3 + TrkBshc- + Grb10</td>
<td></td>
<td></td>
<td></td>
<td>-42.9 ± 3.2</td>
<td>-5.5 ± 1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-45.1 ± 5.6</td>
<td>-5.7 ± 1.5</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-50.2 ± 6.1</td>
<td>-5.6 ± 1.4</td>
</tr>
<tr>
<td>Kv1.3 + Grb10</td>
<td></td>
<td></td>
<td></td>
<td>-29.4 ± 6.1</td>
<td>-5.4 ± 0.5</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>-32.4 ± 7.4</td>
<td>-17.2 ± 11.7</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>-34.2 ± 7.9</td>
<td>-3.4 ± 3.6</td>
</tr>
<tr>
<td>Kv1.3</td>
<td></td>
<td></td>
<td></td>
<td>-28.2 ± 5.8</td>
<td>-4.2 ± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-31.1 ± 5.3</td>
<td>-5.0 ± 0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-31.5 ± 6.8</td>
<td>-4.9 ± 1.1</td>
</tr>
</tbody>
</table>

HEK 293 cells were transfected as treatment conditions shown in the table. Kv1.3 currents were recorded in cell-attached membrane patches held at a holding potential (V_h) of –90 mV and stepped to -5 mV by 5 mV step depolarizations (V_c) with a pulse-duration of 50 ms and 10 ms interpulse intervals between protocol in Table3.1a for a total of 3 times. Repeated measures ANOVA and Tukey-Kramer post-test was used to compare pre (a), during (b) and post (c) stimulation parameters within treatment conditions with significance level at α ≤ 0.05. Significantly different pre, during and post measurements are denoted in parenthesis within the table.

While these experiments confirmed that Kv1.3 can be phosphorylated in the presence of N-Shc we still had not resolved the question of whether it could in some way disrupt the interaction at the membrane between activated TrkB and Kv1.3 that would result in current suppression but we
Figure 3.7 Tyrosine phosphorylation of Kv1.3 channel protein following BDNF stimulation.

A, HEK 293 cells were transiently transfected with Kv1.3 cDNA alone (K alone), or plus TrkB (KT), or plus TrkB and Grb10 (KTG), or plus TrkB and N-Shc or plus TrkB lacking the kinase domain plus N-Shc (KDS). Cells were stimulated for 10 minutes with vehicle control (-) or BDNF (100 ng/ml) (+) in media. Kv1.3 channel protein was immunoprecipitated (IP) from whole-cell lysates, shown in the top panel, and separated by SDS-PAGE. Proteins were electro-transferred to nitrocellulose. The lysates were then blotted (Blot) with anti-Kv1.3 (top panel) and the IP was blotted with anti-phosphotyrosine antibody α-4G10 (anti-pY). The blots were then visualized by species-specific hrp-conjugated secondary antibody. The upper arrow (>) indicates phosphorylated Kv1.3 channel protein and the lower arrow indicates the heavy immunoglobulin band (IgG).

B, HEK 293 cells were transiently transfected with mycKv1.3 cDNA which allows IP of Kv1.3 from the membrane using c-myc antibody. The conditions were mock transfection (Mock), mycKv1.3 + TrkB (mycKT), mycKv1.3 + N-Shc (mycKS), and mycKv1.3 + TrkB and N-Shc (mycKTShc-S).
mycKv1.3 + TrkB + N-Shc (mKTS) and mycKv1.3 + TrkBshc- + N-Shc. Cells were stimulated for 10 minutes with vehicle control (-) or BDNF (100 ng/ml) (+) in 30mm K+ pipette solution. Membrane Kv1.3 channel protein was immunoprecipitated (IP) from cells by incubation of c-myc antibody with intact cells before BDNF stimulation then continuing the IP protocol from the whole-cell lysates. Proteins were separated by SDS-PAGE. Proteins were electro-transferred to nitrocellulose. The immunoprecipitates were then blotted (Blot) with anti-pY (top) stripped and blotted with anti-Kv1.3 (middle) and stripped a second time and blotted with anti-c-myc (bottom). The blots were then visualized by species-specific hrp-conjugated secondary antibody. C, blots of lysate and IP same as in B blotted with anti-SHC.

now know that the level of nShc was not sufficient to totally prevent TrkB phosphorylation of Kv1.3.

On further consideration of the question we realized that perhaps our measure of overall levels on Kv1.3 phosphorylation in the -/+ BDNF conditions would not be sensitive enough to pick up changes we expected to occur with Kv1.3 channels in the membrane. We redesigned our experiment using a myc epitope tagged channel in which myc was placed between the S1 and S2 domain of Kv1.3 such that it remains exposed on the extracellular surface when the channels are inserted into the membrane. We used cmyc antibody to specifically immunoprecipitate only the channels on the surface and then looked at phosphorylation levels of this pool of channels. Due to the low levels of expression of Kv1.3 in the presence of Grb10 we performed these batches of experiments with N-Shc co-expression. The results of these re-designed experiments are shown in Figure 3.7 B. In the first blot the only phosphorylation signal for Kv1.3 is in the lane which contains mycKv1.3 + TrkB + N-Shc. The results were unanticipated because we expected that we would see phosphorylation signals for all the conditions except mycKS (mKS) and mKTshc-S (mKTshc-S) with lower levels of phosphorylation in +BDNF lane of mKTS compared to the +BDNF lane of mKT. On closer examination, the results of these experiments qualitatively suggest that the presence of the adaptor protein N-Shc keeps the channels on the membrane longer allowing us to actually detect a pool of phosphorylated channels in the membrane. Note that the second blot demonstrates that there were channels in the membrane even where there was no phosphorylated signal in the blot above, which was stripped a second time and blotted with anti-cmyc. The blot with anti-cmyc confirms that we only IP surface Kv1.3 and not intracellular myc as there is no signal in the first two lanes. These experiments were repeated a total of 4 times.
**Grb10 interacts directly with Kv1.3.** The expression of Kv1.3 was reduced significantly (Figure 3.8 B) in the presence of Grb10 and even when the TrkB receptor was also present. Note that the co-expression of Grb10 in HEK 293 cells is modest as seen in the first blot in Figure 3.8 A. There are already high levels of Grb10 in HEK 293 cells which is expected as the HEK 293 cell line is an immortalized human embryonic kidney cell line and hGrb-IRβ/Grb10 transcript has been found expressed in kidney tissue from humans (Frantz et al., 1997). We cannot predict what the exact balance of Grb10 levels would be in a transiently transfected cell line but for these experiments we demonstrate the effect of Grb10 on the expression level of Kv1.3.

Given the observation that over-expression of Grb10 reduced Kv1.3 expression (Figure 3.7 A and Figure 3.8 A) as well as relieved BDNF current suppression of Kv1.3 (Figure 3.3 D) we tested whether or not Grb10 can directly interact with Kv1.3. Immunoprecipitation of Kv1.3, Grb10 and TrkB from various combinations of conditions are shown in Figure 3.8 C. We find that Kv1.3 was co-immunoprecipitated from an IP of Grb10 shown in the first IP blot (Figure 3.8 C) suggesting that most of Grb10 exists in a protein-protein interaction with Kv1.3 in the cell. In the reverse IP shown in the last IP blot (Figure 3.8 C) we get a signal matching that for IP Grb10 blot Grb10 when we blot the Kv1.3 immunoprecipitates with Grb10 antibody. Note that we lose the lower band weight of Kv1.3 in this IP, suggesting that the bulk of Kv1.3 in the cell with over-expressed Grb10 may exist mostly in a higher KDa weight form. Grb10 did not co-immunoprecipitate with TrkB in these transiently transfected HEK293 conditions regardless of the level of Grb10 expression in the -/+ Grb10 transfection condition.

**Grb10 lowers surface expression of Kv1.3 without lowering channel current levels.** Despite the observation that over-expression of Grb10 lowered the cellular levels of Kv1.3, recordings of Kv1.3 current from cell-attached membrane patches of HEK 293 cells, transfected with Kv1.3 and Grb10, produced Kv1.3 current that was comparable to the levels obtained in Kv1.3 alone conditions. This implies that there must be adequate amounts of Kv1.3 in the membrane to produce functional tetrameric ion conducting channels. To confirm this, the level of mycKv1.3 channel expression was tested in the presence and absence of Grb10 and immunocytochemical staining done against cmyc in non-permeabilized cells. The presence of Grb10 lowered and dispersed the cmyc signal and cells representative of this effect are shown in
Figure 3.9 A. Examples of the average peak outward current obtained from recordings of Kv1.3 transfected -/+ Grb10 (K, KG), Grb10 + TrkB (KTG) and +TrkBshc-/- Grb10 (KTshc-, KTshc-G) are also shown in Figure 3.9 B, along with a histogram summarizing the mean Peak Current amplitudes for the various conditions.

Figure 3.9 A. Illustration of Grb10 protein-protein interactions with Kv1.3. Lysates from HEK 293 cells transiently transfected -/+ Grb10 alone or + mycKv1.3 (K, KG) or with TrkB -/+ mycKv1.3 (KT, KTG). Lysates from the various conditions were separated by SDS-PAGE then electro-transferred to

Figure 3.8 Grb10 protein-protein interactions with Kv1.3. A, HEK 293 cells were transiently transfected -/+ Grb10 alone or + mycKv1.3 (K, KG) or with TrkB -/+ mycKv1.3 (KT, KTG). Lysates from the various conditions were separated by SDS-PAGE then electro-transferred to
nitrocellulose and blotted (Blot) as shown with anti-Grb10, anti-cmyc and anti-Kv1.3.  B, histogram representing Kv1.3 expression in lysates from conditions as shown above for mycKv1.3 -/+ Grb10 (K, KG) and mycKv1.3 + TrkB -/+ Grb10 (KT, KTG).  C, HEK 293 cells were transiently transfected -/+ Grb10 alone or + mycKv1.3 (K, KG) or with TrkB -/+ mycKv1.3 (KT, KTG).  Lysates from the various conditions as shown and IP of Kv1.3, Grb10 and TrkB from the various conditions were separated by SDS-PAGE then electro-transferred to nitrocellulose and blotted (Blot) as shown with anti-Grb10 for the first three sets of blots.  The last IP was Grb10 blotted with anti-Kv1.3.

Figure 3.9 Surface expression and function of Kv1.3 in the presence of Grb10.  A, HEK 293 cells were transiently transfected with cDNA for mycKv1.3 and mycKv1.3 + Grb10.  Kv1.3 in
the membrane was labeled using anti-cmyc against the myc surface epitope between the extracellular S1 and S2 domains of Kv1.3 in mycKv1.3. B, HEK 293 cells were transiently transfected with cDNA for Kv1.3 (K), Kv1.3 + Grb10 (KG), Kv1.3 + TrkB + Grb10 (KTG), Kv1.3 + TrkBshc- (KTshc-) and Kv1.3 + TrkBshc- + Grb10 (KTshc-G). B also shows histogram representing mean peak outward current for the various conditions.

Discussion

The co-localization experiments done in the olfactory bulb suggest that Kv1.3 can potentially interact with both N-Shc and Grb10 in fiber-like structures in the internal plexiform layer, the granule cell layer and to a lesser degree in some of the fibers surrounding the glomeruli. We believe these fiber-like structures to be axonal or dendritic in nature. The possibility exists that these axonal/dendritic structures may contain exclusively N-Shc or Grb10 or expressed at different levels relative to each other, Kv1.3 and other interacting partners. Most of the studies done looking at distribution of Grb10 has been by Southern, Northern and Westerns Blots using various regions of tissue throughout the body (Frantz et al., 1997; Blagitko et al., 2000). The distribution of N-Shc/ShcC has been similarly studied in various tissues. In this study Shc labeling was found in the mitral cell layer. This work however is the first of its kind in the olfactory bulb showing distribution of Grb10 and how it co-localizes with an ion channel it is now shown with in a protein-protein interaction.

The adaptor proteins N-Shc and Grb10 both contain SH2 domains that allow them to bind to short sequences in proteins containing phosphorylated tyrosines such as phosphorylated Kv1.3. We know that Kv1.3 becomes phosphorylated on BDNF activation of TrkB and this correlates with suppression of the current. One possibility is that co-expression of N-Shc or Grb10 prevents BDNF-induced Kv1.3 current suppression by interacting with the phosphorylated tyrosines on the channel directly and hampering a subsequent event that leads to current suppression. The data suggest this may be the case as there is no gross decrease in phosphorylation level of Kv1.3 by BDNF activated TrkB in the presence of the adaptor protein N-Shc. More importantly, the presence of detectable tyrosine phosphorylated Kv1.3 in the membrane for the KT condition only in the presence of N-Shc suggests that N-Shc does not prevent or disrupt Kv1.3 phosphorylation. This finding provides evidence that N-Shc may in
fact be disrupting an event that happens after phosphorylation of Kv1.3 such as binding to phosphorylated tyrosines. Membrane-inserted Kv1.3 co-immunoprecipitates with N-Shc indicating a protein-protein interaction with N-Shc possibly through the SH2 domain. Co-expressed N-Shc prevents BDNF-evoked current suppression of Kv1.3 suggesting that endogenous levels of N-Shc expression may also act as suppressors of BDNF modulation of Kv1.3 by keeping the phosphorylated channel around longer in the membrane. The effect of Kv1.3 current suppression may involve actual removal of the channel from the membrane and the data here indirectly support this hypothesis. In a series of convincing experiments tyrosine-phosphorylation dependent suppression of Kv1.2 current was proven to be caused by endocytosis of the channel from the membrane (Nesti et al., 2004). However, measurement in Shc⁻/⁻ mutant mice of the tyrosine phosphorylation levels of the NMDA receptor subunits, found both the NR2A and NR2B levels to be increased (Miyamoto et al., 2005). They checked kinase activity of Src family kinase and found no notable difference compared to WT mice and concluded that N-Shc was probably involved in recruiting a certain protein tyrosine phosphatase to the receptor multi-complex. It is possible that N-Shc may regulate Kv1.3 differently to the NMDA receptor or it might be that N-Shc may interact with the phosphorylated NMDA receptor in a similar way to Kv1.3 and hinder an early post-phosphorylation event that leads to increased or prolonged activation of phosphatases.

The TrkB shc site is also important for N-Shc to hinder the post-phosphorylation event that results in current suppression because we do not see phosphorylated channels on the membrane when the shc site is lacking on TrkB. Likewise, the data suggest that the TrkB shc site is also needed for Grb10 to suppress BDNF-induced current suppression of Kv1.3. What is interesting is the more pronounced suppression of about 30% of Kv1.3 current when BDNF activates TrkBshc-. This suggests that the TrkB shc site is involved in interactions that have the effect of lessening the amount of current suppression observed with TrkB WT to values between 16-18%. Over the duration of the effect we did not observe any significant changes in the conductance or other properties of the channel in KT + BDNF from the 5th to the 20th minute. Neither did the mere presence of TrkB or TrkBshc- have an effect on the biophysical parameters of Kv1.3 measured in this study.

However, the ability of cellular molecules to disrupt BDNF induced Kv1.3 current suppression may not depend solely on interactions with phosphorylated Kv1.3 through SH2
domains. PSD95, which has the ability to regulate and cluster Kv channels surface expression and distribution (Tiffany et al., 2000; Wong et al., 2002; Eldstrom et al., 2003), does not require SH2 domains to bind and therefore it is not dependent on interacting only with the phosphorylated channel but still relieves BDNF induced Kv1.3 current suppression. PSD95 can interact with Kv channels because they contain PDZ recognition domains that can recognize and interact with short-sequences of Kv channels carrying the sequence motif ETDV in the C-terminals. Kv1.1, 1.2, 1.3, and 1.5 have been shown to also interact with PSD95 via interactions in the N-termini (Eldstrom et al., 2003). Therefore it is quite possible for structural interactions such as with PSD95 to be the basis for hindering the effect of current suppression and may not require interaction with or disturbance of an interaction with phosphorylated tyrosines like may be the case with N-Shc and Grb10. It is conceivable that PSD95 can also bind to the proline rich regions of Kv1.3 via its SH3 domain. Kv1.3 has rich proline rich regions in both the N and C termini. Grb10 was co-immunoprecipitated with Kv1.3 in the absence of TrkB. In the absence of TrkB the levels of basal Kv1.3 tyrosine phosphorylation, in HEK 293 cells, is almost undetectable so it seems plausible to conclude that Grb10 and Kv1.3 can form a protein-protein interaction that may not depend on SH2 interactions. This does not exclude the ability for Grb10 to bind to Kv1.3 via SH2 interactions when BDNF activation of TrkB leads to tyrosine phosphorylation of Kv1.3. Apart from hindering possible removal of Kv1.3 from the membrane Grb10 and PSD95 may be relieving current suppression by altering a biophysical property of Kv1.3. This occurred in the Kv1.3 + TrkB condition where current suppression was disrupted with Grb10 and PSD95. During the 23 minutes of acute BDNF stimulation for Kv1.3 + TrkB + Grb10 and Kv1.3 + TrkB + PSD95 the $V_{1/2}$ of Kv1.3 was left-shifted significantly. The Kv1.3 + TrkB + Grb10 condition also had a speeding of the inactivation.

An unexpected finding was that Grb10 can reduce the expression and alter the distribution pattern of Kv1.3 without changing the channel current levels in the membrane. Although we have not measured current to capacitance ratio we are recording in the cell attached configuration and use electrodes with negligible variability in the diameter and resistance of the pipette tips (see methods). Hence, we are of the opinion that the capacitance changes from one membrane patch to another will not significantly impact on our result. Grb10 is capable of regulating ion channel expression, it was found to regulate the epithelial channel by the ubiquitin ligase Nedd4 and ubiquitination (Staub et al., 2000). Coincidently Nedd4 co-expression with
Kv1.3 in oocytes decreases Kv1.3 current (Morrione et al., 1999) and mGrb10 interacts with Nedd4 in a yeast two-hybrid system (Morrione et al., 1999). Given that Grb10 co-immunoprecipitates with Kv1.3 in HEK 293 cells, Grb10 may cause ubiquitination of Kv1.3 thereby regulating channel expression in the cell and on the membrane. It is possible that the higher kDa Kv1.3 band observed in the presence of Grb10 may be ubiquinated Kv1.3. HEK 293 cells may express Nedd4 and this may be an obvious question to answer next in investigating the mechanism whereby Grb10 down-regulates the expression of Kv1.3. Grb10 has also recently been found to mediate insulin-stimulated degradation of the insulin receptor (Ramos et al., 2006). The recent discovery that Kv1.3 plays a part in insulin signaling (Xu et al., 2004) and regulates GLUT4 trafficking to the membrane (Li et al., 2006) along with interaction of IR and Grb10, Kv1.3 and Grb10, the relationship between IR, Kv1.3 and Grb10 warrants further investigation.

Overall these findings suggest that the expression levels of adaptor proteins and PSD95 may very well modulate BDNF effects on Kv1.3. It is possible that they may have overlapping mechanisms that depend on N-Shc and Grb10 SH2 interactions with phosphorylated tyrosines of Kv1.3. However they may also be the result of direct interaction with channel at other sites for instance PSD95 and Kv1.3 may interact via PDZ domains of PSD95 with the C or N termini of Kv1.3. Kv1.3 and N-Shc and Kv1.3 and Grb10 have specific overlapping patterns within the olfactory bulb that suggests the importance of their relationships for olfactory bulb function particularly a novel function of Grb10 which co-immunoprecipitates with Kv1.3. Co-expression of Grb10 also results in down-regulation of Kv1.3 and re-distribution of surface expression without altering function. An important next step would be to see if the expression profile of N-Shc and Grb10 change with development, levels of activity and injury in the olfactory bulb. It would also be interesting to see if chronic BDNF stimulation can alter the expression levels of these downstream molecules and change membrane excitability of those neurons by altering the properties of Kv1.3.
CONCLUSION

Understanding exactly how the cell can use kinases such as TrkB to strategically alter the electrical properties of the membrane by regulating Kv channel expression level and subcellular-distribution is paramount in the potassium ion channel field to contribute to fully understanding changes in excitability and plasticity of neurons as well as during development and injury. In the olfactory bulb, BDNF and the downstream signaling of its activated receptor TrkB may have a classical role as a neurotrophic factor enhancing and supporting the survival of mature olfactory receptor neurons as well as a neuromodulator involved in plasticity. BDNF can suppress Kv1.3 current in both rat and mouse olfactory bulb neurons. This dissertation has explored the effects of BDNF and its receptor on the function and regulation of Kv1.3. It has shown that BDNF can acutely suppress Kv1.3 current by phosphorylating key tyrosine residues YYY111-113FFF, Y137F, Y449F. It has demonstrated that TrkB kinase activity can result in the increase of expression and current levels of Kv1.3 by increasing the stability of the protein in the cell. It has also shown that the replacement of tyrosine residues 111-113 to phenylalanines reduces the expression of Kv1.3 while loss of function results in an increase of expression such as with the W386F non-conducting Kv1.3 pore mutant. However, interestingly TrkB decreases Kv1.5 expression while the insulin receptor decreases Kv1.3 expression but has no effect on Kv1.5 expression indicating that the activity of TrkB and IR may be used differentially in response to stimuli depending on the needs of the cell.

The co-localization of TrkB with Kv1.3 in neuronal fibers (axons and/or dendrites) and to a lesser extent in the intraglomular glomerular structures lends credence to BDNF functioning, as suspected, as a neuromodulator of electrical excitability in the olfactory bulb. Under which olfactory stimuli might they be chiefly used? There are numerous possibilities mainly because the fibers passing through the granule layer are in an area containing fibers from higher brain regions that can act to prime activity within the mitral cell layer as they make connections to granule cells and mitral cells (Wilson, 2003). The granule cells are the most numerous cells of the olfactory bulb and are GABAergic cells (Stewart et al., 2002). They make their contacts onto
the output neurons of the olfactory bulb, mitral and tufted cells, and are very important for this first level processing of odorant stimuli. The significance of finding TrkB and Kv1.3 in the region is two fold therefore because it is a place where BDNF can have survival, maturation and plasticity roles both in the developing olfactory bulb and in maintenance of circuits. The granule and periglomerular cells of the olfactory bulb are continuously replenished via progenitor cells from the subventricular zone travelling into the olfactory bulb via the rostral migratory stream (Luskin, 1993; Zigova et al., 1998). These neurons receive inhibitory inputs from granule cells via dendrodendritic reciprocal synapses, consisting of an excitatory mitral-to-granule cell synapse directly adjacent to an inhibitory granule-to-mitral cell synapse (Price and Powell, 1970). The mitral cells relay synchronous input from many different olfactory receptor types to single cells and diverse populations of neurons in the piriform cortex. Piriform cortical pyramidal cells, in turn, make extensive associational connections throughout the piriform cortex, back to the olfactory bulb as well as to other cortical structures. The relationship of modulation between Kv1.3 and TrkB and possibly even IR can play a crucial role in translating experience into plastic changes in the olfactory bulb and cerebral hemispheres and also perhaps in other biological functions such as microglial activation. Potassium current levels change during development of mature neurons and circuitry in the brain and after stroke and injury. Hence, the olfactory bulb represents a unique opportunity to study the relationship between the Kv1.3 ion channel and neurotrophic and neuromodulatory factors such as BDNF.

In Chapter three, the effects of over-expression of the adaptor proteins N-Shc and Grb10 was investigated. These findings are important for understanding the physiological significance of adaptor protein interactions for the outcome of BDNF modulation of Kv1.3 and perhaps other ion channel targets in the brain that can have implications for neuronal excitability and membrane changes involved in development, maturation and survival of neurons and their circuits. It also has specific importance for understanding adaptor protein and TrkB function in the olfactory bulb and any role of the TrkB receptor in the olfactory bulb that may overlap or compete with the function of the insulin receptor. TrkB and IR can activate overlapping downstream end points via their SH2 domains and PI3/Akt kinase (Zhao et al., 1999; Whitehead et al., 2000; Barnabe-Heider and Miller, 2003; Mizuno et al., 2003; Dou et al., 2005). This would expand the possible ways that tyrosine kinases can alter the electrical response patterns of neurons such as mitral cells in the olfactory bulb where Kv1.3, TrkB and IR are found. It means
that one pathway may have precedence over another depending on the type of stimuli or they may allow receptor specific responses to be activated at the same time. Insulin signaling plays an important role in differentiation and survival and so does TrkB. TrkB and BDNF signaling apart from their known and classical roles in survival are also important for plasticity and learning in various parts of the brain. This dissertation has contributed to understanding how TrkB, BDNF and downstream signaling molecules can utilize basic biochemical features of proteins such as Kv1.3 to modify their properties and regulate their expression in the neuron. Kv1.3 is found predominantly expressed in the main output neurons of the olfactory bulb called mitral cells and also in the largest population of neurons in the olfactory bulb, the granule cells. The granule cells are inhibitory in nature with connections in the olfactory bulb that places their dendrites in strategic locations for shaping odor detection and meaning. In addition, the olfactory bulb is one of the few structures in the brain that provides the opportunity to study perpetual re-integration of new neurons into existing circuitry. This dissertation shows the promise for the future exploration of Kv1.3, and its role in, neuronal survival, integration into and sculpting of existing circuitry in the olfactory bulb.
APPENDIX A

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VIA E-MAIL.

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